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Local Degranulation of Individual Rat Peritoneal Mast Cells Induced by Compound 48/80

By

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Abstract

DIAMANT, B, P G KRUGER and B UVNÄS *Local degranulation of individual rat peritoneal mast cells induced by compound 48/80* Acta physiol scand 1970 79 1—5

Degranulation of individual rat mast cells is shown to occur at the site of administration of compound 48/80 by the use of micropipets placed close to the mast cell membrane Varying degree of degranulation can be induced It is possible to induce repeated degranulation by local application of 48/80 to the same area of the cell at least four times The results suggest that compound 48/80 acts directly on the cell membrane and this interaction seems to be the primary cause of the extrusion of granules

The morphology of isolated rat mast cells has been thoroughly studied since there seems to be an intimate link between morphological changes and histamine release from these cells The extrusion of granules (commonly denoted as degranulation) from mast cells exposed to histamine releasing agents was first recognized by Riley and West (1953) Various mechanisms for the histamine releasing process have been proposed Thon and Uvnäs (1967) pointed to a possible two stage process, a primary energy requiring transport of histamine-containing granules to the outside of the mast cell and a secondary ion exchange of granule histamine for extracellular cations

Horsfield (1965) demonstrated with cinephotomicrography that compound 48/80 induced granules to pass through the mast cell membrane by a process involving the formation of large vacuoles through the fusion of smaller perigranular ones and the sudden shrinkage of the cell Horsfield (1965) and Bloom and Haegermark (1965) suggested from electronmicroscopic investigations that the perigranular membrane fused with the cell membrane before the granular material would leave the cell A mechanism similar to reversed pinocytosis was discussed in this connection The extracellular granules from rat mast cells are no longer surrounded by a membrane (Bloom and Haegermark 1965) In analogy with the unifying theory of Robertson (1966) relating cell structure to various problems of cell function, Fillion *et al* (1969) discussed the possible extrusion of histamine carrying granules from intracellular canaliculi harbouring the granules and opening to the surface of the cell

The mechanism by which compound 48/80 induces degranulation of mast cells is not known Although graded to the dose of compound 48/80 and sometimes involving the extrusion of only a few granules the mast cell response both *in vivo* and (in cell suspensions) *in vitro* occurs diffusely spread over the mast cell surface It is not known whether the degranulation requires a penetration of compound 48/80 into the mast cell or if it is the result of an action of the releaser on membrane sites It was thought possible to answer this question using local microapplication of compound 48/80 to the mast cell surface

Methods and materials

Isolated rat peritoneal mast cells were placed under a Leitz Ortoplan microscope in a hanging drop in an oil chamber of acrylic plastic at 37° C as described by Diamant and Krüger (1968) Micropipets with tip diameters ranging between 1—2 μ were filled with compound 48/80 with a concentration of ,—1 μg/ml The tips of the pipets were placed close to the mast cell surface and 48/80 was delivered by pressure from a syringe filled with paraffin oil and connected by a plastic tubing to the pipet

The microscope was used with a plan apochromat oil immersion lens 100/1 32 and a 0 60/1 11 condensor

Compound 48/80 was generously supplied by Dr B Hogberg AB Leo Halsingborg Sweden

Results

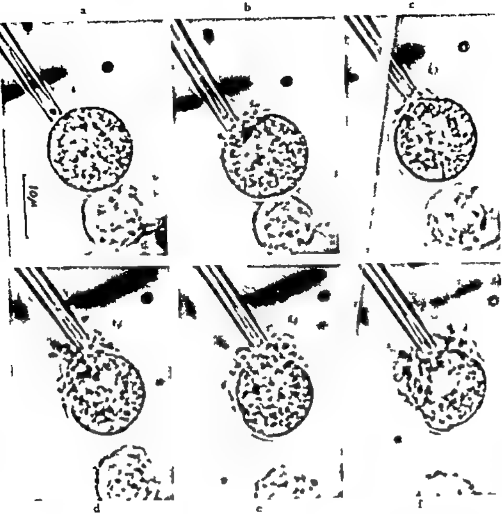
A single mast cell with a pipet is shown in Fig 1 a After application of 48/80 to the surface of the cell a local degranulation was induced which seemed to start immediately upon administration of 48/80 (Fig 1 b 4 sec after application) The degranulation process did not spread spontaneously to other areas of the cell than those originally affected (Fig 1 c 30 sec after application of 48/80) A second dose of 48/80 at the same site of the cell surface caused further extrusion of granules (Fig 1 d 48/80 applied 90 sec after the first application) It was possible to repeat this local degranulation by administration of 48/80 on the same area of the cell at least four times Fig 1 e 48/80 applied 120 sec and Fig 1 f 150 sec after the first application)

In Fig 2 a mast cell is shown after application of 48/80 from the same pipet at two different sites of the membrane by turning the cell around between applications It is evident that degranulation induced locally did not render the rest of the cell insensitive to 48/80

Depending on the amount of 48/80 delivered the area of the mast cell that became degranulated could be varied to involve only a few square microns up to the entire cell surface

Discussion

A parallelism has previously been observed between the number of mast granules released from mast cells in situ and the amount of histamine liberated after i p injection to rats of Tyrode solution containing increasing concentrations of compound 48/80 (Fawcett 1954) A similar parallel dose response curve between degranulation and histamine release was obtained by Fillion *et al* (1969) on exposure of isolated rat mast cells *in vitro* to compound 48/80 The present results show that the mast cell



F . . . C . . . mpo nd 48/80 5 μg/ml a prior to
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fter fourth applica

Fig 2 Degranulat on caused by appl ca t on of compound 48/80 at two different s tes of the mast cell surface

surface will respond to 48/80 with a local degranulation wherever a certain minimal concentration of the degranulating agent is reached Degranulation which is known to occur within seconds when mast cells are incubated with 48/80 (Uvnas and Thon 1961, Horsfield 1965 Bloom Fredholm and Haegermark 1967) is equally fast with the method used in the present investigation In fact with the present method no apparent lag period was noted between application and cellular response

The morphological changes by 48/80 could be induced to involve only a limited area of the cell surface In no case was a spontaneous spreading of the degranulation process from the initially degranulated area observed It seems therefore that 48/80 exerts a direct local effect on the cell membrane initiating a process that results in the extrusion of granules In addition a mast cell could be induced to degranulate repeatedly upon repeated local application of 48/80 Cinephotographic studies have revealed that repeated application of 48/80 to the same site of the mast cell caused additional extrusion of granules by the involvement of adjacent non affected areas of the cell membrane By the present method it is not possible to determine if in addition 48/80 induced the extrusion of new granules from already affected areas of the cell

The present observations support the view that degranulation does not represent a mechanism which will induce irreversible damage of the mast cell and are in agreement with biochemical observations that a soluble enzyme like lactic dehydrogenase *does not leak out nor does endogenous ATP of the mast cells decrease after degranu*lation and histamine release induced by 48/80 (Diamant 1967 a b) These findings were recently confirmed by Johnson and Moran (1968) who in addition showed that 48/80 treated cells retained acid phosphatase as well as most of the K^{42} which was allowed to accumulate prior to the addition of 48/80

Furthermore previous studies have shown that mast cells incubated with 48/80 will after proper washing of the cells respond with additional degranulation and histamine release by a second exposure to 48/80 without intervening washing however the cells remain refractory to further exposure to 48/80 (Thon and Uvnas 1967) Thus the available information points to the fact that isolated rat mast cells stay viable after degranulation induced by 48/80

Diamant and Kruger (1968) reported on the effect of local application of ATP on the morphology of mast cells No degranulation was observed and the reversible configurational changes of the cell membrane that occurred took place after a longer period of exposure than the degranulation response to 48/80 The present findings therefore stress the difference between the mechanisms behind histamine release induced by ATP (Diamant and Kruger 1967 and 48/80

The present findings suggest that a mast cell should be looked upon as a cell which can release small numbers of granules known to contain histamine serotonin heparin and proteolytic enzyme This may be taken to support the old view that mast cells have a physiological function in the body acting as unicellular glands (Ehrlich 1880 Arnold 1906 Stammler 1921 Nagaso 1928) The demonstration that a mast cell is able to repeatedly release granular material upon stimulation suggest that the

cell might exert a humoral influence on smooth muscle tone v somotor activity capillary permeability etc

It is not known if mast cells when exerting physiological fu tions release their histamine through a degranulating or a non degranulating cell ar process In fact nothing definite is as yet known regarding those factors w ch might regulate histamine release from mast cells *in vivo* The intimate contact tween the mast cell membrane and the capillary endothelium demonstrated in elec nmicroscopic studies by Flood and Kruger (1969) suggest however, that blood rn or local humoral factors might influence histamine release under physiologica well as pathological conditions

This study was supported by the Swedish Medical Research Co l (B68 14X 40-04C and K67 14X 1004 02) the National Institutes of Health Bethesc 4 Md USA (2 RO1 AM04063 00 and 5 RO5 TW00306-03) and the Scientific Re e Council of Norway E 1130 1)

References

ARNOLD J Zur Morphologie und Biologie der Mastzellen l ocyten und Lymphocyten *Munch med Wschr* 1906 53 585—589

BLOOM G D and Ö HAEGERMARK A study on morphological anges and histamine release induced by compound 48/80 in rat peritoneal mast cells *I Cell Res* 1965 *40* 637—654

BLOOM G D, B FREDHOLM and Ö HAEGERMARK Studies c he time course of histamine release and morphological changes induced by histamine l ators in rat peritoneal mast cells *Acta physiol scand* 1967 *71* 270—282

DIAMANT B, Observations on some metabolic enzymes of th ast cell and the macrophage

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DIAMANT B and P G KRUGER Structural changes of isol ted rat peritoneal mast cells induced by ATP and ADP *J Histochem Cytochem* 1968 *16* 707—716

EHRLICH P Metodologische Beitrage zur Physiologie und Pathologie der verschiedenen Formen der Leucocyten *Z klin Med* 1880 *1* 553—560

FAWCETT D W Cytological and pharmacological observat ons on the release of histamine by mast cells *J exp Med* 1959 *100* 217—223

FILLION G M B S A SLORACH and B UVNAS The release of histamine heparin and granule protein from rat mast cells treated with compound 48/80 *in vitro Acta physiol scand* 1970 *78* 547—560

FLOOD P R and P G KRUGER Fine structure of mast cells in the central nervous system of the hedgehog To be publ 1969

HORSFIELD G I, The effect of compound 48/80 on the rat mast cell *J Pat Bact* 1965 *90* 599—605

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rol (Lond)

ROBERTSON J D, Certain concepts of cell structure relevant to release of neurohumoral agents In *Mechanism of Release of Biogenic Amines* Pergamon Press 1966 3—27

STAMMLER M, Untersuchungen uber Vorkommen und Bedeutung der histogenen Mastzellen im menchlichen Korper unter normalen und pathologischen Verhaltnissen *Frankfurt Z Path* 1921 25 391—435

T itive steps in the *1* 303—315

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Acta physiol. scand. 1970. 79. 6—23

From the Department of Physiology, University of Göteborg, Sweden

Characteristics of Receptors and Afferent Fibres of the Forelimb Interosseous Nerve of the Cat

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Abstract

SILFVENIUS, H. *Characteristics of receptors and afferent fibres of the forelimb interosseous nerve of the cat.* Acta physiol. scand. 1970. 79. 6—23.

A description is given for dissection and identification of the forelimb interosseous nerve, Int f, a branch of the median nerve. The nerve contained on average 225 axons. The measured diameters of the myelinated axons of the Int f ranged between 1 and 16 μ. A bimodal distribution of fibres was found with peaks around 4 μ and 10 μ. The fibres of the low threshold main component of the compound action potential conducted at velocities between 85 and 110 m per sec. The properties of receptors and afferent fibres were studied in 52 single fibres of the median nerve. About 50 per cent of these were rapidly adapting, the others slowly adapting. Among the rapidly adapting 33 per cent were low threshold vibration sensitive Pacinian receptors whose fibres conducted at velocities between 63 and 117 m per sec. The remaining [illegible] per cent were identified as high threshold tap receptors, some of which were vibration sensitive. Their fibres conducted at velocities between 27 and 118 m per sec. 36 per cent of the slowly adapting units were tension receptors with fibres conducting at velocities between 27 and 110 m per sec. The other group (12 per cent) consisted of muscle spindles. They conducted at velocities between 8[illegible] and 110 m per sec. About 4 per cent of the population remained unidentified. Functional aspects of the findings are discussed.

Knowledge of the nature and origin of the afferent input to a defined locus is a step on the way on understanding of central nervous mechanisms. Central responses evoked by *stimulation of receptors and afferent nerves have therefore often been* studied and graded electrical stimulation of afferent nerves has in many investigations been considered to be the method of choice. This method may be used for selective activation of afferent axons with similar thresholds to electrical stimulation, i.e. with similar axon diameters and conduction velocities, but an interpretation of such results requires a knowledge of the receptors which are subserved by the selected afferents.

In a recent series of investigations (Landgren, Silfvenius and Wolsk 1967a and b, Landgren and Silfvenius 1969) it was considered of interest to study the cortical projections of the Pacinian afferents from the cat's fore- and hindlimb. Such afferents

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are abundant in the interosseous nerves. Due to the work of H nt and McIntyre (1960) and Barker (1962) the receptors with axons in the inte seous nerve of the hindlimb (Int h), are relatively well known Skoglund (1960 as shown that the interosseous nerve of the forelimb (Int f), contains afferent t es from rapidly as well as from slowly adapting receptors For the Int f., howev the correlat on between axon diameters conduction velocities and receptor type has not been stud ed in detail Such an investigation was therefore carried out and results are presen d in the following report. A preliminary note has been publi he Silfvenius 1968

Methods

15 cats were used. Anesthesia was induced by vaporizing 2 % alothan Hoechs in a Fluotec apparatus into a 2 1 mixture of oxygen nitrous oxide T anesthesia was ma ntain ed with chloralose 70 mg/kg given i v supplemented at times doses of 3 % Nembu a (Abbott) i v B oodpressure rectaltemperature and the tempe e of the nerve pool were monitored. The pool temperature was kept around 36—37° C. T ight forelimb was mounted to a metal ring stand. The mounting allowed the performanc passive movements of the limb The tendons of the flexor muscles were cut at the carpus better exposure

The Int.f was exposed via a medial skin incision. Whi e inta nt f was tested w th electrical stimulation of high strengths no movements or twitches e observed The nerve wa then cut proximally to the receptor cluster of the interosseous e All other branches of the median nerve were cut and the median nerve was cut prox lly on entering the brach a plexus

The cut Int.f was prepared for electrical stimulation by p ting its distal end on a pair of chlorided silver electrodes. Two pairs of similar recording ctrodes were moun ed on the median nerve The compound action potential was recorded r ophasically from the proxima pair and triphasically from the distal one At the end of the eriment the nerve was cut at the distal recording position and a monophasic recording ma there The length of the nerve between these two positions was measured The threshold he compound action potential evoked by a 100 µsec square wave pulse was determined. T atency of the compound potential was measured and the conduction time between the two ording positions was calcula ed The compound action potential was photographed as recor f superimposed traces Stimulus escape to nearby branches was not thought likely to occur nce the threshold of the evoked potential was safely below that of a potential evoked in the nerve after Int f had been pinched proximally to the stimulating electrode C-fibres were not udied

In the studies on Int.f receptors and their single atte nts the median nerve was meticulously dissected. Tiny branches from the nerve were f nd to proceed to the elbow joint region and to the periosteum of the humerus They were ut because receptors located there could be activated by the tests performed. Impulse discharges evoked in Int.f by mechanical activation of its receptors were recorded from microdissected strands of the median nerve close to the brachial plexus The strands were hooked on a silver wire electrode whose reference electrode was nearby A pair of stimulating electrodes was hooked under Int.f The distance between the stimulating cathode and the recording electrode was measured and the conduction velocities were calculated from conduction time and distances. The unitary po entials from fast adapting receptors were recorded on the CRO monitored on a loudspeaker and directly photographed. Discharges from slowly adapting units were taped by a Revox recorder Sequential analyses of the taped discharges were made with an Intertechnique Didac 800 Physioscope with a discriminator Its pulse input triggered a control CRO fed with the original sp kes from the Revox. This provided a reliable observation of the triggering level The stored data was registered over the analogue output on a Hewlett Packard X—Y recorder

Stimulation of rapidly adapting receptors was carried out with an electrodynamic mechanical stimulator A perspex rod was attached to the metal pin of the stimulator diaphragm. A 17 mm long steel pin with a blunt tip (Ø 0 4 mm) was drilled into the distal end of the rod After the receptor site had been approximately identified the tip of the stimulator pin, attached to a stereotaxic apparatus was adjusted under microscopic control to make contact w h he ... we

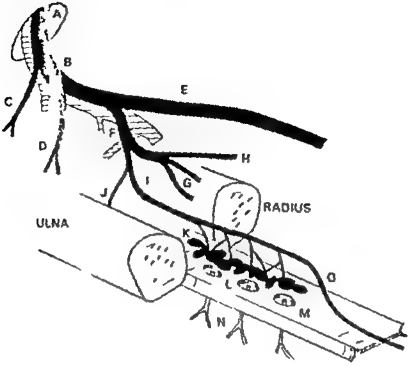

Fig 1 D s e t on d ag am tl e left med an nerve
Through tl e supra o d d foramen (A tl e med an nerve B pa es togetl er witl fo e mb ves el l at l ed D ollowing nervebranches are nd cated
C bran h to p onato te muscle D branch to palmaris longus tl e flexor carpi ra l al s m scles an l to tl e I III ads of FDP E superf cial sk l ranch F deep l ranch d v ng d between IV V FDP l ves off l ranch G to IV V FDP and l ran l H to PQ Int f erve J p ox mal anch of Int f procee l ng prox mally in tl e interros eous space Pa n an e ep o u te l nteros eous membrane M Membranel oles tl ro gl wl cl essel N penet ate O d a branch of I t f to carpal jo nt o perl aps furtl er d stall

l e IV an l V l ead IV V of tl e flexor d g torum profundus muscle FDP Str ngs were ed to tl e l tal en l IV V FDP Pull ng and load ng was done from tl e strings Tl e receptors we e teste l l appl g tl e st mula or t p on tl e receptor region and use t ng a tea ly pres u e D wa e t ant f ed Detail o tl e metl ods of act at on a e g en n the results

Tl e pulse shape and tl e a t de of tl e st mulator output was mon tored w tl a transducer ampl f er em tl a prov d a long t me-constant w tl respect to tl e pulses use l Tl e d placements of tl e t mulat were cal l rated opt cally 40 x magn f c for botl s ngle an l for s nuso dal pul es at d ff t frequencies Tl e accura y of tl e cal l rat ons was of tl e order of 10 % Tl e m l tor l wed a l near relat on h p between np t an l o tput voltages By extrapolat ng f n t e d ed voltages tl e peak to-peak ampl tu les of the d placements could be asses e l S mul a ous mon tor ng of tl e transd cer outp t voltages made it pos l le to cl oose a l n a n a a pl tudes of des re l magn u les w tl n known l m ts Tl e t me-constant of tl e ep x pulse del vered to tl e electrodynam c st mulator was 1 4 msec S nuso dal st m la wa p ided l y a Pl l ps Au l ogenerator Tl e respon e of tl e receptors to Brevid l l Suxetl on brom le May & Baker Ltd was s ud ed l y nject ng 100 µg/kg of tl e drug tl e fe oral ve n Art f c al ventilat on w tl 96 O_2 was g ven as requ red

The axon d ameters f In f were ea u ed af er l per ce t OsO_4 sta n ng accor l ng to tl e method of Rexe l (1944 Tl e clu e f Pac n an receptors was st l ed in a few preparat ons us ng e tl er OsO_4 — or an C esom ta n ngs Sect ons were prepared af er embedd ng n paraff n

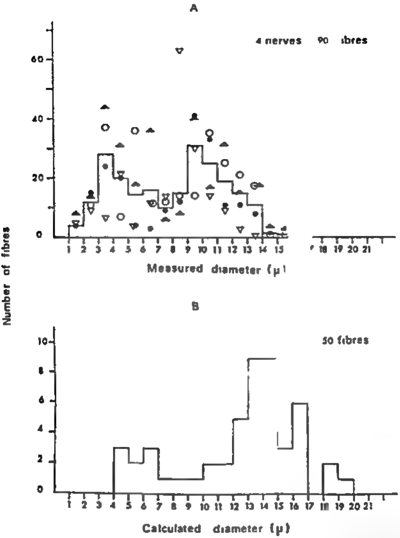

Fig 2 Axon diameter distribution of Int f In A are shown the results from measurements ... stribution in individual ... in the diametergroups ... B is shown the axon ... The axondiameters of the two unidentified units are not included in diagram B

Results

Anatomical and histological findings Int f is a branch of the median nerve Fig 1 (B) The median nerve passes through the supracondyloid foramen (A) of the humerus to the antebrachium Distal to the foramen the nerve is covered by the pronator teres muscle which is supplied with a branch (C) After this other branches (D) are given off to the palmaris longus the flexor carpi radialis muscles and to the

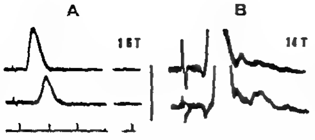

Fig 3 The compound action potential of Int f obtained from two separate experiments In A is the velocity of the low threshold fastest conducting fibres 105 m per sec calculated from monophasic recordings at two different sites 44 mm apart on the median nerve, orthodromic stimulation In B high intensity stimulation was used to demonstrate the component of delta fibres The fast conducting main low threshold component is off It is comprised of fibres with conducting velocities between 52 and 89 m per sec as measured from recordings 13 mm apart The threshold of delta wave was 6T the fastest delta fibres conducted at 29 m per sec antidromic stimulation Numbers to the right in A and B indicate stimulus strengths used Voltage bars 200 µV Time scale 1 msec Positivity downward

I—III FDP A skin branch (F) proceeds superficially Midway between the elbow and the carpus the median nerve sends a branch (F) deep inbetween the IV—V FDP This branch then divides One of the divisions supplies the IV—V FDP (G) and proceeds (H) to the pronator quadratus muscle, PQ

The other division is Int f (I) It accompanies the interosseal vessels giving off its branches to the proximal part of the interosseous space (J and other not drawn in Fig 1 Int f proceeds distally in the space At the bottom of the space the interosseous membrane (L) As Int f proceeds it fans out into thin strands many of which come from Pacinian corpuscles (K) which are clustered in the space The receptor cluster is in contact with the IV—V FDP, but also with the membrane If the cluster is elevated holes (M) are seen in the membrane Through these branches of the interosseal vessels penetrate to the extensor digitorum communis muscle Penetrating nerves were not found on histological examinations of two specimens If PQ was split a nerve branch (O) was found to proceed distally towards the carpal joint or perhaps even further

Histological sections showed 30 to 40 Pacinian corpuscles in the cluster Others were dispersed as solitary ones or appeared in small groups in the region The corpuscles were easily distinguishable under the dissection microscope and appeared as typical lancet shaped greyish bodies the large ones being about 800 µ long (cf Quilliam and Sato 1955

The axon diameter spectrum of Int f was investigated in four histological preparations Int f had a diameter ranging from 175—200 µ (mean 190 µ) and held between 195—296 myelinated axons (mean 225) The diameters of the myelinated axons ranged from 1 to 16 µ The distribution of the measured axon diameters is given in Fig 2A The histogram suggests a bimodal distribution with peaks near 10 and 4 µ. Such a distribution is also indicated by the histogram of axon diameters computed according to the approximation of Hursh (1939) from the observed conduction velocities in 50 single Int f fibres Fig 2B The peaks are, however, found

TABLE I Stimulation strengths required to evoke a maximal response, and the conduction velocity of the compound action potential of Int f in 5 expts

Main component			Delta component	
Strength required for a maximal response (multiples of threshold strength (T))	Conduction velocity of fastest fibres m/s	Conduction velosity of slowest fibres m/s	Threshold of delta fibres T	Conduction velocity of fastest delta fibres m/s
2 7	110	77	—	
1 8	105	80	10	
2 4	85	57	—	
2.2	108	64	7	
2 6	87	52	6	29
Average 2 3	97	66	8	29

at somewhat higher diameter values in the latter histogram This may be due to methodological errors in either technique

The compound action potential of Int f

The compound action potential recorded from the median nerve in response to graded electrical stimulation of Int f is shown in Fig 3 The main component of the compound action potential was generally a smoothly rising and falling potential without indentations Occasionally, however, a step was seen near the peak but no distinct separation was gained by the technique of Bradley & Eccles (1953) In record A of Fig 3 the monophasically recorded main compound action potential is shown The voltage necessary to activate the largest fibres ranged between 0 04—0 09 V Maximal amplitude of the main component potential was reached at 1 8—4 2 (mean 2 3) times threshold T At stimulus strengths of about 8 T a late delta component was evoked (Fig 3B)

The observed conduction velocities are given in Table I The average velocity of the fastest fibres of the main component of the compound action potential was 97 m per sec and the corresponding value for the slowest fibres in this component was 66 m per sec The conduction velocities of the fastest fibres in the delta component as measured in one experiment, was 29 m per sec

Unitary analysis of receptors and afferent fibres of Int f

A Rapidly adapting receptors

I Low threshold vibration sensitive mechanoreceptors, Pacinian corpuscles

A population of 52 functional single unit preparations of the median nerve was investigated The units were all discharged by electrical stimulation of Int f and the conduction velocities of the axons determined Two units remained unidentified 17

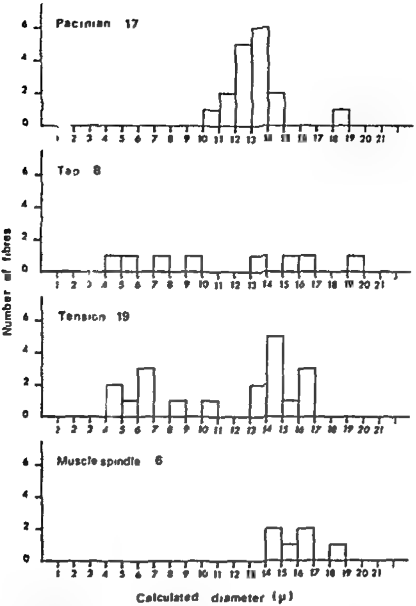

Fig. 6. Histograms of Int f. based on calculated axon diameters of afferents of the four mechanically identified receptors of Int f. A histogram sum is shown in Fig. 2B.

on the amplitude of the stimulating pulses. At optimal frequency following the amplitude was indeed low. In record F of Fig. 4 it may be seen that a minimum amplitude of 5 μ was sufficient for a 1 : 1 ratio at 400 cps. Higher and lower frequencies required an increase of the pulse amplitude. The difference in amplitude required to discharge the unit at various frequencies is further illustrated in Fig. 4 H–K. In H the frequency is 200, in I 120 and in K 70 cps. The unit ceases to respond at

fading amplitudes of 9, 17 and 35 μ respectively The relations be een the frequencies of the vibration and the threshold amplitude of a 1 1 resp /stimulus ratio is further illustrated in Fig 5 Between frequencies of 150 and cps the action potentials followed sinusoidal vibrations of very low amplit , mean 11 μ A minimum pulse amplitude was found between 300 and 400 cps a mean threshold amplitude lower than 4 μ At stimulating frequencies higher an 500 cps, larger amplitudes of the order of 50 μ were required Below 100 the threshold amplitudes increased markedly Other response/stimulus ratios also observed

The conduction velocities observed in the afferent axons of Pacinian corpuscles varied from 63 to 117 *m per sec* The axons were thus large w calculated diameters above 10 μ The distribution of the axon diameters is show Fig 6 which shows the distribution of the axon diameters of the four different oups of receptors described in the present report

II High threshold units, tap receptors

In their investigation of the hindlimb interosseous nerve H t and McIntyre (1960) classified units with a high threshold and a fast adaptati rate as tap receptors Similar properties were found in 8 units *i e* in 15 4 per t of the Int f receptors These units were discharged only by strong tapping direc on radius or ulna or on the metal support of the limb They were not dischar by tapping on the paw These receptors responded with only one action potent Plucking strings of tissue in the interosseous space was an effective way of activat g the units Such plucking performed with a glass rod was in fact the method u for localization of the receptors The stimulus was obviously complex It presu ably delivered a sharp pull to the tissue, followed by vibration of the tissue Mechanical pulses of rather large amplitude (80—540 μ) applied to the site where pl cking evoked a response discharged some of the units which also followed vibrating stimuli of rather high amplitude generally in the low frequency range In apparent contrast to the Pacinian units, the tap receptors did not respond to pressure with a glass rod in the interosseous space or elsewhere Supinatory/pronatory or flexor/extensor movements of the carpal joint or of the digits did not discharge the units

It was possible to localize the receptive areas of five tap units These areas were located within the interosseous space One receptor responded to plucking the interosseous nerve vessels bundle within a restricted area less than 10 mm long Plucking from the ulnar side of the bundle was more effective than plucking from the radial side and lowering the tension of the bundle by appropriate positioning of the limb lowered the threshold of the unit to mechanical stimulation Another receptor could be activated by plucking on tissue strings within an area of 5—7 mm Its receptive area was in the proximal part of the interosseus space One tap receptor was located in the middle of the cluster of Pacinian corpuscles Its receptive area was about 5 mm in diameter Finally the receptive areas of two receptors were found just proximal to PQ Three of the localized receptors responded to mechani-

60 cps was ap ied to the receptor site The discharge followed the vibrations This particular unit esponded to 60 100 and 220 cps but failed to follow intermediate frequencies in ie range between 20 and 800 cps even with high amplitude pulses The response thirteen tension receptors was tested with intravenous injection of Suxethonium)0 μg per kg) The units were allowed to adapt to a moderate stretch before the injection No change in the frequency of the discharge was observed in 11 of t units In the remaining two a decrease occurred which resulted in a cessation of the discharge after a time lapse of 40—50 seconds from the beginning of the injection When stretching was increased the receptors resumed their discharge This ob ation as well as the rather long latency of the effect suggests that the cessation of th lischarge was due to relaxation of the muscle

In early experim a slowly adapting receptor was localized to the carpal joint capsule covering the region the triquetrum and hamatum bones It responded to dorsiflexion of the paw This was tl only observation of a joint receptor with its axon travelling in Int f Th conduction veloc it was not determined

The conduction ve ocity of the afferent fibres from the tension receptors varied from 27 to 100 m per sec The group thus consisted of axons with a diameter from 5 to 17 μ Most of the fibres (11 out of 19) were, however, large having diameters above 14 μ (c) Fig 6

II *Muscle spindles*

Six slowly adapting unit i e 11 5 per cent of the population were classified as muscle spindles The criteria u ed for their classification were, their characteristic response stretch with a pronounced dynamic component the pause in the discharge during e contraction of the muscle their sensitivity to vibration and the increase in dis harge in response to injection of Suxethonium The muscle spindles were activated by movements of the limb as were the tension receptors Three units responded to supination and one to pronation of the limb One unit was mainly activated by dorsiflexion of the carpus The units responded to palpation with a glass rod and to stretch of PQ and of IV V FDP They were therefore easy to localize One was found in PQ three in V and two in IV head of FDP They were located proximally in the heads because cutting of FDP at about 10 mm above the insertion did not interfere with the response to pulling of the remaining muscle stump

The typical response of a muscle spindle to maintained stretch is shown in Fig 8 A A 60 g load was applied to IV FDP The short lasting high frequency dynamic component characteristic of muscle spindles (Matthews 1933) was found in all the FDP units

The silent period in the discharge during contraction of the muscle may be seen in record B The contracti n was induced by electrical stimulation of the muscle itself, at strength below 2 × twitch threshold Cessation of the discharge was also observed during contraction evoked by electrical stimulation of the muscle nerve Prior to the pause in record B there is an increase in impulse discharge in response to muscle contraction This may reflect irregularities in contractile events

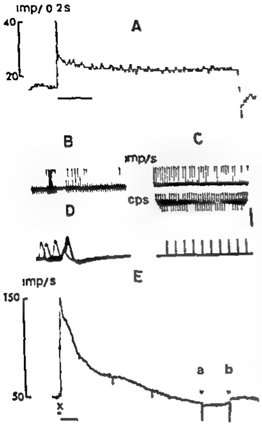

Fig 8 Record rom a muscle spindle located in IV 1 DP, 4 mm proximal to PQ The unit ponded to stretching of IV FDP but d not in paw movements

A response a 60 g load B response to isometric c raction Length of silent period in B at 50 msec adapted frequency 88 in per sec C upper record response to soidal vibration at receptor site IV 1 P being loaded with 60 g Lower recor stimulation frequency 430 cps amplit 43 μ D action potentials evoked by trical stimulation of Int f at 2 F C ction distance 99 mm E sequential stogram obtained during intraveno injection of Suxethonium (100 μg k IV FDP loaded with 60 g Line unde x indicates injection Arrows at a and indicate recording pauses of 2 and 1 m lengths

Time les A 10 sec B 50 msec, C 10 c D 0 5 msec E 20 sec Voltage 100 μV

All the muscle spindle units were discharged by sinusoidal vibration (see Fig 8C) They followed vibration frequencies in a 1 1 ratio up to between 100 to 500 cps (mean 300 cps) The lowest amplitudes used for activation varied from 13 to 215 μ The stimulus was usually applied to the muscle in close vicinity of the receptor A response was obtained both from loaded and unloaded muscles but frequency following generally improved if the muscle was stretched (*cf* Granit and Henatsch 1956 Bianconi and van der Meulen 1963 Brown Engberg and Matthews 1967)

Five units were tested with intravenous injections of Suxethonium (100 μg per kg) All of them responded with a marked increase in discharge frequency with a latency of about 5 sec after initiation of the injection This observation is in accordance with the reports of Granit Skoglund and Thesleff (1953), and Rack and Westbury (1966) describing the increase in discharge of muscle spindles in response to succinyl choline

Record E of Fig 8 shows the response to Suxethonium of a unit located in FDP The IV FDP was loaded with 60 g There occurred a prompt increase in frequency lasting for about 3 min With the muscle unloaded the duration of increase in discharge frequency was generally about 1 min The conduction velocity of the afferent

60 cps was ap[plied] to the receptor site The discharge followed the vibrations This particular unit [r]esponded to 60, 100 and 220 cps but failed to follow intermediate frequencies in [the] range between 20 and 800 cps even with high amplitude pulses The response [of] thirteen tension receptors was tested with intravenous injection of Suxethonium [(10]0 μg per kg) The units were allowed to adapt to a moderate stretch before [the] injection No change in the frequency of the discharge was observed in 11 of [the] units In the remaining two a decrease occurred which resulted in a cessation of th[e d]ischarge after a time lapse of 40—50 seconds from the beginning of the injection [W]hen stretching was increased the receptors resumed their discharge This obs[erv]ation as well as the rather long latency of the effect suggests that the cessation of th[e] discharge was due to relaxation of the muscle

In early experim[ents] a slowly adapting receptor was localized to the carpal joint capsule covering the region [of] the triquetrum and hamatum bones It responded to dorsiflexion of the paw This was [the] only observation of a joint receptor with its axon travelling in Int.f The conduction velo[city] was not determined

The conduction velocity of the afferent fibres from the tension receptors varied from 27 to 100 m per sec. The group thus consisted of axons with a diameter from 5 to 17 μ Most of the fibres (11 out of 19) were, however, large having diameters above 14 μ (*cf* Fig 6

II Muscle spindles

Six slowly adapting unit[s] *i e* 11 5 per cent of the population were classified as muscle spindles The criteria used for their classification were, their characteristic response [to] stretch with a pronounced dynamic component, the pause in the discharge during the contraction of the muscle, their sensitivity to vibration and the increase in discharge in response to injection of Suxethonium The muscle spindles were activated by movements of the limb as were the tension receptors Three units responded to supination and one to pronation of the limb One unit was mainly activated by dorsiflexion of the carpus The units responded to palpation with a glass rod and to stretch of PQ and of IV—V FDP They were therefore easy to localize One was found in PQ three in V and two in IV head of FDP They were located proximally in the heads because cutting of FDP at about 10 mm above the insertion did not interfere with the response to pulling of the remaining muscle stump

The typical response of a muscle spindle to maintained stretch is shown in Fig 8A A 60 g load was applied to IV FDP The short lasting high frequency dynamic component characteristic of muscle spindles (Matthews 1933) was found in all the FDP units

The silent period in the discharge during contraction of the muscle may be seen in record B The contraction was induced by electrical stimulation of the muscle itself, at strength below 2 × twitch threshold Cessation of the discharge was also observed during contraction evoked by electrical stimulation of the muscle nerve Prior to the pause in record B there is an increase in impulse discharge in response to muscle contraction This may reflect irregularities in contractile events

sumption Another part of the class has large axons but the receptors are located in the periosteum As they are easily localized it should be possible to obtain a histological identification but until this has been achieved their classification as tendon organs remains unestablished Some of the tension receptors have axons in the delta range They are therefore not compatible with Group I b axons in muscle nerves and the morphology of the tension class is thus heterogeneous Whether or not this also holds for their function remains to be investigated

The muscle spindles form a small but homogenous and w t identified group of receptors Their identification in Int f rests on functional observations Their axons are large the receptors are located near the insertion of the muscle and act in parallel with the extrafusal fibres They also respond to Suxethonium A larger number of spindles were seen to be present in Int f than in Int h and they can hardly be regarded as aberrant in this situation as was thought by Hunt and McIntyre (1960) for Int h

Int f was found to innervate muscle spindles and tension receptors located near the insertion of the muscles suggesting that certain muscles may have Group I afferents which are not accounted for by an investigation of the motor nerves

Although some additional information of the tap receptors was gained in this study the exact nature of the receptors and their morphology remains obscure Barker (1962) did not describe any endings of Int h that would fit these receptors They may be functionally impaired Pacinian units or constitute a separate group of high threshold endings or perhaps receptors whose adequate stimulation has yet to be defined At variance with the findings of Hunt and McIntyre (1960) the present experiments showed that the receptors responded to vibration although pulses of relatively large amplitudes were required The wide range of observed conduction velocities suggests that the tap receptor group is heterogenous

Considerable interest has been focused on the role played by vibration sensitive afferents in sensory mechanisms Talbot *et al* 1968 Merzenich and Harrington (in press) Whether or not the Pacinian afferents also are involved in motor mechanisms has so far attracted little interest There are however studies on man on the effect of vibration on motor performance which may be relevant in this context The contraction which the vibration of a muscle elicites has been attributed to the excitation of the primary endings (*cf* Hagbarth and Eklund 1966 1968 Lang and Vallbo 1967) Similar effects were however also seen when joint regions were vibrated Such effects could be interpreted as being mediated by activation of other vibration sensitive endings than the spindles *i e* the Pacinian corpuscles These have been found in joint regions (Gray & Matthews 1951 Skoglund 1956 Hromada and Poláček 1958 Burgess and Clark 1969) and in the surrounding of ligaments and tendons Because of their extreme sensitivity to bone conducted vibrations Pacinian receptors located even further away could take part in the effects observed The possibility should therefore be considered that activation of Pacinian or paciniform receptors could contribute to the motor effects seen How these receptors are involved in reflex patterns remains to be investigated

What functional role could then be attributed to the Pacinian receptors of the interosseous region? Should they be considered to participate mainly in sensory mechanisms as mediators of touch and vibration (Talbot *et al.* 1968) or could they also convey information which would be utilized in motor performance? Since the receptors are clustered in such an abundance in the 'closed' interosseous space, they are likely to be mechanically activated by different movements of the limb. Hunt and McIntyre (1960) have, in fact, demonstrated that the Pacinian receptors are discharged during muscle contraction. No doubt during volume alterations of the interosseous space the receptors would respond, such alterations would occur when pronator/supinator, flexor/extensor, and adductor/abductormuscles contracted. Due to their sensitivity and rapid adaptation the Pacinian corpuscles would signal start and change of movement. They would therefore provide only a rather crude type of information. The great number of receptors and their arrangement in the interosseous space would, however, provide a quantification of afferent discharge. During movement of the limb a mechanical distortion will travel through the tissue and affect the different corpuscles in a temporal and spatial sequence characteristic of the movement. The magnitude of the distortion which acts upon the individual corpuscles may also vary in a characteristic way. The result could be a pattern of afferent discharge unique for the movement.

The present study was undertaken in order to obtain the information necessary for a use of graded electrical stimulation of the interosseous nerves in the analysis of central nervous mechanisms. It is obvious from the findings that four functionally different receptors have afferents in the lowest threshold component of these nerves. The origin of centrally evoked responses must therefore be interpreted with caution. As it is true that the interosseous nerves provide a large sample of Pacinian afferents it should therefore be possible to differentiate responses evoked by electrical stimulation of these afferents from those of the other receptors. This could be achieved by comparing the electrically evoked responses with those evoked by direct mechanical stimulation of the cluster of Pacinian corpuscles.

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References

Barker, D. The structure and distribution of muscle receptors. *In Symposium on Muscle Receptors.* Ed. D. Barker. Hongkong University Press 1962. 227—240.

Bianconi, R. and J. P. van der Meulen. The response to vibration of the end organs of mammalian muscle spindles. *J. Neurophysiol.* 1963. *26.* 177—190.

Bradley, K. and J. C. Eccles. Analysis of the fast afferent impulses from thigh muscles. *J. Physiol.* (Lond.) 1953. *122.* 46—473.

Brown, M. C., I. Engberg and P. B. C. Matthews. The relative sensitivity to vibration of the muscle receptors of the cat. *J. Physiol.* (Lond.) 1967. *192.* 772—800.

Burgess, P. R. and F. J. Clark. Characteristics of knee joint receptors in the cat. *J. Physiol.* (Lond.) 1969. *203.* 317—335.

Granit, R. and H. D. Henatsch. Gamma control of dynamic properties of muscle spindles. *J. Neurophysiol.* 1956. *19.* 356—366.

GRANIT R ▪ S SKOGLUND and S THESLEFF Activation of muscle spindles by succinyl choline and decamethonium The effects of curare *Acta physiol scand* 1953 *28* 134—151

GRAY J A B and J L MALCOLM The initiation of nerve impulses b mesenteric Pacinian corpuscles *Proc roy Soc B* 1950 *137* 96—114

GRAY J A B and P B C MATTHEWS Response of Pacinian corpuscles in the cat s toe *J Physiol* (Lond) 1951 *113* 475—482

HAGBARTH K E and G EKLUND Motor effects of vibratory muscle muli in man *In Proceedings of the First Nobel Symposium* Ed R Gran t Stockholm Almqvist and Wiksell 1966 177—186

HAGBARTH K E and G EKLUND The effects of muscle vibration i spasticity, rigidity and cerebellar disorders *J Neurol Neurosurg Psychiat* 1968 *31* 20 213

HROMADA J and J POLACEK, A contribution to the morphology of ▪ apsulated nerve endings in the joint capsule and in the periarticular tissue *Acta anat* (Basel) 1958 *33* 187—202

HUNT C C and A K MCINTYRE Characteristics of responses fron receptors from the flexor longus digitorum muscle and the adjoining interosseous region of t cat *J Physiol* (Lond) 1960 *153* 74—87

HURSH J B Conduction velocity and diameter of nerve fibers A er *J Physiol* 1939 *127* 131—139

LANDGREN S and H SILFVENIUS Projection to cerebral cortex Group I muscle afferents form the cat s hind limb *J Physiol* (Lond) 1969 *200* 353 2

LANDGREN S H SILFVENIUS and D WOLSK Somato-sensory ; ths to the second cortical projection area of Group I muscle afferents *J Physiol* (Lond 967 a *191* 543—559

LANDGREN S H SILFVENIUS and D WOLSK Vestibular cochle and trigeminal projections to the cortex in the anterior suprasylvian sulcus of the cat *J ysiol* (Lond) 1967 b *191* 561—573

LANG A H and A B VALLBO Motoneurone activation by low tensity tetanic stimulation of

a Pacinian corpuscle *J gen*

MATTHEWS B H C Nerve endings in mammalian muscle *J Physiol* (Lond) 1933 *78* 1—53

MERZENICH M M and T HARRINGTON The sense of flutter ibration evoked by stimulation of the hairy skin of primates comparison of human sensory capacity with the responses of mechanoreceptive afferents innervating the hairy skin of monkeys *Exp Brain Res* (In press)

RACK P M H and D R WESTBURY The effects of suxamethonium and acetylcholine on the behaviour of cat muscle spindles during dynamic stretching and during fusimotor stimulation *J Physiol* (Lond) 1966 *186* 698—713

REXED B Contributions to the knowledge of the postnatal development of the peripheral nervous system in man *Acta neurol belg* 1944 Suppl 33

QUILLIAM T A and M SATO The distribution of myel n on nerve fibres from Pacinian corpuscles *J Physiol* (Lond) 1955 *129* 167—176

SATO M Response of Pacinian corpuscles to sinusoidal vibration *J Physiol* (Lond) 1961 *159* 391—409

SCOTT D JR Response of Pacinian corpuscle to oscillatory stimulation *Fed Proc* 1951 *10* 123

SILFVENIUS H Vibration receptors in the forelimb of the cat *Acta physiol scand* 1968 *73* 5A

SKOGLUND C R. Properties of Pacinian corpuscles of ulnar and tibial location in cat and fowl. *Acta physiol scand* 1960 *50* 385—386

SKOGLUND S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 Suppl 124

TALBOT W H I DARIAN SMITH H H KORNHUBER and V B MOUNTCASTLE The sense of flutter vibration comparison of the human capacity with response patterns of mechanoreceptive afferents from the monkey hand *J Neurophysiol* 1968 *31* 301—334

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Afferent Fibres in the Hypoglossal Nerve of Cat

By

Jerker Hanson and Lennart Widen

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Abstract

Hanson J and L Widen *Afferent fibres in the hypoglossal nerve of cat* Acta physiol scand 1970 79 24—36

Electrical stimulation of the hypoglossal nerve in cat elicited bilateral twitching of the vibrissae and suppression of shivering The afferent fibres which mediate these reflexes have a higher stimulus threshold than the motor fibres They travel to the brain stem via anastomoses between the hypoglossal nerve and the vagus group in the region of the ganglion nodosum Unitary activity was recorded in the spinal trigeminal nucleus after stimulation of the hypoglossal nerve The reflexes are probably nociceptive reactions since similar reflexes can be evoked from the greater part of the body surface by pain stimuli Stimulation of a peripheral branch of the hypoglossal nerve also elicited a local reflex response in the styloglossus muscle The reflex is polysynaptic Any tonic background activity in the muscle is inhibited by the stimulation It is shown that this inhibition is probably not recurrent but mediated by afferent fibres via anastomoses to the vagus group Afferent impulses evoked by stretching the tongue were recorded in the proximal part of the hypoglossal nerve trunk These units behaved like muscle spindles In cat these units are sparse and probably of secondary importance for movements of the tongue

The question of whether the hypoglossal nerve consists of motor fibres only or also contains sensory fibres is disputed Among those who ascribe the nerve sensory fibres opinions differ as to whether these travel with the nerve into the medulla oblongata or via anastomoses cross over into other nerves Conditions appear to differ in different animal species but the results of investigations in the same animal species are also conflicting In the following brief survey stress is laid mainly on investigations in cat A more comprehensive review has been published by Blom (1960)

Anatomical studies

A root ganglion upon the hypoglossal nerve has been described in cat by Froriep and Beck (1895) Langworthy 1924 a b) and Holliger (1955) although is was not encountered in all animals examined Tarkhan and Abd-el Malek (1951) found sensory nerve cells along the nerve trunk mainly in the distal half they were usually scattered and very few in number in one cat however a ganglion with 200 cells was found

Connections between the trunk of the hypoglossal nerve and cervical ganglia have been found in some mammals but not with certainty in the cat (Hinsey and Corbin

1934 Corbin and Harrison 1938 Downman 1939) On the other hand peripheral anastomoses have been clearly demonstrated between the hypoglossal and lingual nerves (Fitzgerald and Law 1958 Blom 1960) lying on the surface of the styloglossus muscle Lingual nerve fibres have not been described in the mor proximal parts of the hypoglossal nerve

According to textbooks of anatomy (e g Clara 1959) there ex ts in man a connection between the vagus groups and the hypoglossal nerve whe these nerves lie in proximity to each other at the level of and proximal to th ganglion nodosum These connections are assumed to contain autonomic fibres I is uncertain whether these accompany the hypoglossal nerve peripherally to the ongue muscles Boyd (1941) was unable to demonstrate this anastomosis in rabb On the other hand Tarkhan and Abou el Naga (1947) found in dog a small n mber of degenerating fibres in the hypoglossal nerve after removal of the gangl n dosum We have been unable to find any information regarding conditions in the it

Physiological studies

Several attempts have been made to record afferent impu l in the proximal part of the hypoglossal nerve trunk during various types of stim ation to the cat s tongue (Barron 1936 Corbin and Harrison 1938 Downman 193 Blom 1960 Porter 1966) but with negative results Cooper (1954) and Blom (196 however found impul e activity in the peripheral end branches of the nerve stretching the tongue but along which path these impulses are propagated to th brain stem has not been clarified Neither Cooper (1953) Law (1954) nor Blon 1960) were able to demonstrate muscle spindles in the intrinsic muscles of the c t s tong e and it is therefore unknown what type of stretch receptor is activated

With intracellular recording from the hypoglossal motor neurones Green and Negishi (1963) and Porter (1965 1966) found in addition to antidromic action potentials also postsynaptic potentials in response to stimulation of the central cut end of the nerve Green and Negishi considered these indicated the presence of afferent fibres in the hypoglossal nerve whereas Porter presumed that they were the result of stimulus spread to the lingual nerve

Other indices of afferent fibres in the hypoglossal nerve of cat have been reported Electrical stimulation of the central cut end of the nerve caused rise of blood pressure (Tarkhan 1936) and dilatation of the pupils (Downman 1939) Downman left it an open question whether these reflexes are transmitted via the hypoglossal nerve or the vagus accessory group but Tarkhan and Abou el Naga (1947) who however at the time were working with dogs maintained that the afferent fibres reach the brain stem via the vagus nerve Recently Sauerland and Mizuno (1968) described a hypoglosso-laryngeal reflex mediated by fibres which at the ganglion nodosum pass over into the vagus nerve and travel with this to the brain stem

The present electrophysiological studies show that the hypoglossal nerve contains afferent probably small myelinated fibres which mediate reflex respon es not earlier demonstrated on stimulation [illegible]

have the same course as those described by Sauerland and Mizuno Further it is shown that afferent impulses elicited by stretching the tongue can by recorded in the proximal part of the hypoglossal nerve

Methods

Experiments were performed on 44 adult cats of either sex Most of the animals were under light pentobarbitone (Nembutal, Abbott) anesthesia during the experiments In a few instances chloralosed cats were used or cats decerebrated under pentobarbitone anesthesia by section of the brain stem at the midcollicular level after which further anesthetic was not given

Chlorided silver electrodes were used for stimulating and recording from the peripheral nerves steel needle electrodes for the electromyogram Microelectrodes of stainless steel made according to the method described by Green (1958) were used for recording from single units in the brain stem During these recordings the cat's head was firmly fixed in a stereotaxic instrument (Baltimore Instrument Co) and a large occipital decompression was made in order to decrease the pulsations The stereotaxic coordinates for recording from the spinal trigeminal nucleus were calculated according to Snider's and Niemer's atlas (1961)

Electrical stimuli were delivered from a Grass Model S 8 stimulator with isolation units Unless otherwise stated the stimuli consisted of single shocks of 0 2 msec duration The action potentials were led to an amplifier (Grass Model P 9) connected to a loudspeaker and when necessary combined with a cathode follower The responses were displayed on an oscilloscope (Tektronix Model 502 and photographed with a Grass camera In some experiments a tape recorder (Epsilon Labcorder 0 5000 Hz) was employed and photographs taken during play back

Blood pressure respiration and shivering were recorded on a Grass polygraph shivering in addition on the oscilloscope The transducers used were for blood pressure a Statham strain gauge manometer for respiration a thermistor attached close to the opening of the tracheal cannula and for shivering a capacitive transducer (Dickinson 1950 p 111—116) which recorded movements of the foot In some animals the EMG was recorded with needle electrodes inserted into the m. quadriceps femoris

The site of the microelectrode was marked with the Prussian blue method. The brains were perfused in situ with a mixture of potassium ferrocyanide and formalin and serially sectioned after removal further fixation in formalin and imbedding in paraffin The sections were stained with toluidine blue for microscopic investigation

Results

I *Reflexes evoked by stimulation of the hypoglossal nerve*

Stimulation of the hypoglossal nerve elicited various reflex effects which can be divided into the following groups

A *Effects in reflex systems not directly related to the function of the tongue*

1 *Bilateral movement of the vibrissae*

During dissection of structures in the neck of the cat it was observed that pinching or section of the hypoglossal nerve produced twitching of the vibrissae This movement could also be elicited by electrical stimulation of the hypoglossal nerve (Fig 1 A) and was found to be a reflex discharge in m quadratus labii superioris At stimulus strengths well above threshold for the reflex the latency showed 10—15 msec variations in any one cat and still larger variations between different cats However the latency was never shorter than 20 msec or longer than 55 msec (mean approx 35 msec) At a stimulus frequency of $\leqslant 1$/sec the duration of the response varied between 10 and 60 msec depending among other factors on the depth of anesthesia and the excitability of the preparation The response was usually bilateral generally with a slightly lower stimulus threshold in the contralateral intact muscle

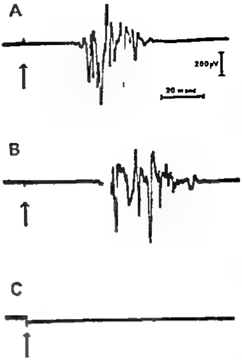

Fig. 1. *A*, Reflex response in m. quadratus labii superioris to electrical stimulation (arrow) of the central cut end of the contralateral hypoglossal nerve. *B*, Same response after intracranial section of the nerve close to the brain stem. *C*, Intracranial section of the roots of the vagus group abolished the response, stimulus strength increased to ensure that no response could be elicited.

Afferent reflex arc. In order to ascertain whether the impulses were transmitted to the brain stem via the hypoglossal nerve or via anastomoses between this nerve and some other nerve, the hypoglossal nerve was sectioned intracranially at its exit from the brain stem. As shown in Fig. 1 B, this had no effect on the response. Likewise ipsilateral section of the trigeminal nerve intracranially in Meckel's cavity, section of the C_1 to C_4 roots or section of the spinal root of the IX nerve had no influence on the response. On the other hand, section of the IX and X nerves intracranially, thus proximal to the ganglia of these nerves, abolished the response (Fig. 1 C).

In order to locate the connections found between the hypoglossal nerve and the vagus and glossopharyngeal nerves, the hypoglossal nerve was dissected free from the periphery in a central direction, dissection being interrupted at intervals to stimulate the nerve immediately distal to its point of intersection with the hyoid bone. Dissection of the hypoglossal nerve at its intersection with the vagus nerve and the ganglion nodosum regularly abolished the reflex response. In other experiments it was found that section of the hypoglossal nerve distal to its intersection with the vagus nerve, *i.e.* at its exit from the bony canal, had no effect on the reflex response. From this it is concluded that afferent fibres travel in the peripheral part of the hypoglossal nerve from its exit from the intrinsic tongue muscles and thereafter join the vagus group at the level of the ganglion nodosum or somewhat proximal to it. At the site of intersection the nerves are surrounded by a good deal of connective tissue and it is impossible to see the anastomoses with the naked eye, neither was it possible to identify them with certainty under the dissection microscope.

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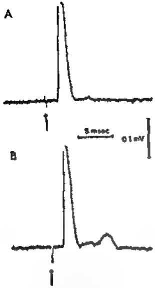

Fig 4

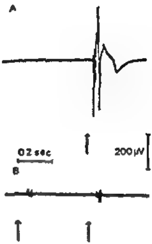

Fig 5

Fig 4 Stimulating and recording from the saphenous nerve Conduction distance between stimulating and recording electrodes 104 mm Stimulus strength in *A*, half the threshold and in *B*, just above threshold for the facial reflex

Fig 5 Conditioning experiment *A*, Reflex response in m quadratus labii superioris to test stimulus to the proximal cut end of the contralateral hypoglossal nerve *B* Same response but strongly inhibited by a preceding stimulus to the contralateral saphenous nerve just supra liminal for the reflex Shock interval about 0.4 sec Arrows indicate stimulus

Type of fibre The reflex response, both the ipsilateral and the contralateral was not elicited until the stimulus strength greatly exceeded the threshold for the direct muscle response in the tongue Fig 3 shows that the action potential from large fibres in the hypoglossal nerve was almost maximal before the threshold for the reflex was reached It has not been possible to demonstrate in the compound nerve action potential any special component which could be ascribed to the fibre group that mediates the reflex The reason for this may be that the conduction distance between the stimulating and recording electrodes was too short (max 40 mm) and that the afferent fibres were too few to give rise to a distinct elevation in the compound action potential

The stimulus threshold however renders it probable that the afferent fibres in the reflex arc are smaller than the motor fibres in the hypoglossal nerve Moving the stimulating cathode 20—30 mm along the hypoglossal nerve caused no measurable change in the latency of the reflex response It is therefore unlikely that the afferent fibres are unmyelinated, they probably belong to a group of small myelinated fibres The long latency appears to be due mostly to the central reflex time, indicating long internuncial chains

Fig 6 Inhibition of shivering in the ipsilateral hindlimb induced by repetitive stimulation (15 stimuli) of the proximal cut end of the hypoglossal nerve Arrows indicate beginning and end of stimulation

It was found that a similar bilateral reflex movement of the cat's vibrissae, due to contraction of mm quadrati labii superioris, could be elicited with pain stimuli, such as pinching the skin with forceps or pricking with a needle, from a large receptive area comprising in excitable animals practically the entire body surface, in less excitable animals *restricted to the head Touch or pressure stimulation of the skin or* passive movements of the limbs never evoked the reflex response

Electrical stimulation of the saphenous nerve in the hind leg evoked a bilateral reflex response in m quadratus labii superioris with a latency about 15 msec longer than that evoked by stimulation of the hypoglossal nerve in the same animal Simultaneous recording of the compound action potential from the saphenous nerve in these experiments (Fig 4) shows that the threshold for the reflex response was not reached until the stimulus strength produced an almost maximal Aβ-spike and a well-developed deflection corresponding to an impulse wave in a fibre group whose fastest fibres had a conduction rate of about 15 m/sec, *i e* belonged to the delta group

In order to determine whether the impulses from the saphenous nerve are mediated to the facial nucleus via the same pathway as the impulses from the hypoglossal nerve a conditioning experiment of the type illustrated in Fig 5 was done When a stimulus to the saphenous nerve just supraliminal for the reflex response preceded stimulation of the hypoglossal nerve the reflex response to the latter was reduced by more than 95 % during a stimulus interval of up to about half a second From this and similar experiments it is concluded that the impulses from the saphenous and hypoglossal nerves before reaching the facial nucleus converge on the same postsynaptic structures somewhere in the brain stem and that the reflex arc is partly the same in both cases

2 *Suppression of shivering*

In cats with spontaneous "shivering" repetitive shock stimuli to the hypoglossal nerve induced suppression of shivering after a latency of almost one second (Fig 6) The effect disappears after intracranial section of the vagus group

Similar suppression of shivering can be obtained with pain stimuli (pinching the skin with forceps) to the limbs or trunk but not by touch pressure or passive movements of the limbs

Fig 7 Reflex response in the styloglossus muscle evoked by a stimulus to the proximal cut end of a branch of the ipsilateral hypoglossal nerve

3 *Effect on blood pressure and pupils*

In cats under pentobarbitone anesthesia and in cats decerebrated under anesthesia stimulation of the central cut end of the hypoglossal nerve produced no unequivocal effects on the blood pressure The pupils were unaffected In cats under chloralose anesthesia however, a rise in blood pressure (about 20 mm Hg) and pupillary dilatation were regularly obtained which is in accordance with the findings of Downman (1939)

B *Local reflexes related to the function of the tongue*

1 *A hypoglosso hypoglossal reflex in the m styloglossus*

Electrical stimulation of the central cut end of a peripheral branch of the hypoglossal nerve elicited a reflex response in the styloglossus muscle after a latency of about 15 msec (Fig 7) The threshold for this reflex response which is abolished by intracranial section of the vagus group is about half the threshold for the reflex response from m quadratus labii superioris

The styloglossus muscle is supplied by the hypoglossal nerve (Barnard 1940 Clara 1959 p 344) a finding we have confirmed by demonstrating that all activity in the muscle is abolished after section of the hypoglossal nerve at its point of exit from the brain stem Accordingly the reflex represents a hypoglosso hypoglossal reflex

2 *Inhibition of tonic activity in extrinsic muscles of the tongue*

The stimulation of the hypoglossal nerve described in the foregoing induced in addition suppression of the tonic activity readily elicited in the extrinsic muscles of the tongue by holding the cat s mouth wide open (Fig 8 A) The suppression occurs after a latency of 10—15 msec and lasts about half a second Blom (1960) who observed this suppression ascribed it to recurrent inhibition The suppression was abolished by section of the vagus group of nerves at their point of entrance into the brain stem (Fig 8 B) which shows that it is mediated by fibres which cross over from the hypoglossal nerve to the vagus group

The threshold for the suppression is about the same as the threshold for the reflex response in m styloglossus but the suppression can however sometimes be induced without the reflex response and accordingly is not secondary to it

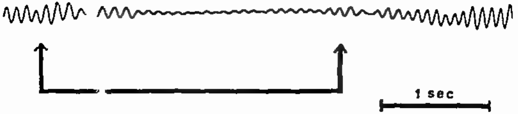

Fig 6 Inhibition of shivering in the ipsilateral hindlimb induced by repetitive stimulation (15 stimuli) of the proximal cut end of the hypoglossal nerve Arrows indicate beginning and end of stimulation

It was found that a similar bilateral reflex movement of the cat's vibrissae, due to contraction of m. quadrati labii superioris could be elicited with pain stimuli, such as pinching the skin with forceps or pricking with a needle, from a large receptive area comprising in excitable animals practically the entire body surface, in less excitable animals restricted to the head Touch or pressure stimulation of the skin or passive movements of the limbs never evoked the reflex response

Electrical stimulation of the saphenous nerve in the hind leg evoked a bilateral reflex response in m quadratus labii superioris with a latency about 15 msec longer than that evoked by stimulation of the hypoglossal nerve in the same animal Simultaneous recording of the compound action potential from the saphenous nerve in these experiments Fig 4) shows that the threshold for the reflex response was not reached until the stimulus strength produced an almost maximal $A\beta$ spike and a well developed deflection corresponding to an impulse wave in a fibre group whose fastest fibres had a conduction rate of about 15 m/sec *i e* belonged to the delta group

In order to determine whether the impulses from the saphenous nerve are mediated to the facial nucleus via the same pathway as the impulses from the hypoglossal nerve a conditioning experiment of the type illustrated in Fig 5 was done When a stimulus to the saphenous nerve just supraliminal for the reflex response preceded stimulation of the hypoglossal nerve the reflex response to the latter was reduced by more than 95 % during a stimulus interval of up to about half a second From this and similar experiments it is concluded that the impulses from the saphenous and hypoglossal nerves before reaching the facial nucleus converge on the same postsynaptic structures somewhere in the brain stem and that the reflex arc is partly the same in both cases

2 *Suppression of shivering*

In cats with spontaneous shivering repetitive shock stimuli to the hypoglossal nerve induced suppression of shivering after a latency of almost one second (Fig 6) The effect disappears after intracranial section of the vagus group

Similar suppression of shivering can be obtained with pain stimuli (pinching the skin with forceps) in the limbs or trunk but not by touch pressure or passive movements of the limbs

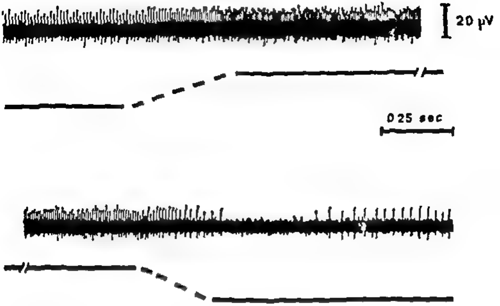

Fig. 9. Unit activity in a filament from the hypoglossal nerve 3 mm proximal to the intersection of the nerve and the carotid artery. Stretching of the tongue is indicated by upward shift of the lower line.

receptor for about one third of a second followed by tonic activity of relatively low frequency. Injection of 15—20 µg/kg of succinylcholine iodide into the external carotid artery in this and another experiment produced a strong increase (>200 %) of the impulse activity without stretching of the tongue.

Active contraction of the intrinsic tongue muscles produced by electrical stimulation through needle electrodes inserted into the tongue induced a "silent period" (Fig. 10 A). On the other hand, contraction of the muscles in the floor of the mouth caused no receptor silence (Fig. 10 B). If the receptor was a stretch receptor, which its behaviour indicates, it seems highly probable that it was situated in some intrinsic muscle and was unloaded on its shortening.

Discussion

The present investigation has shown that the hypoglossal nerve in cat contains afferent fibres which, in the vicinity of the ganglion nodosum, unite with the nerves of the vagus group. In addition, impulse activity from stretch sensitive receptors has been demonstrated in the proximal part of the hypoglossal nerve.

The factors which establish the presence of afferent fibres in the hypoglossal nerve and which have been demonstrated in this study can be divided into three groups, which we consider can most suitably be discussed separately.

1. *The facial reflex, suppression of shivering and effects on blood pressure* were induced at approximately the same stimulus strength and were mediated via

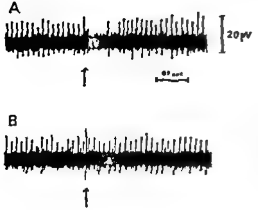

Fig 10 Same experiment as in Fig 9 *A*, The receptor becomes silent during contraction of intrinsic muscles, produced by a stimulus through needle electrodes inserted in the tongue *B*, A stimulus applied to the floor of the mouth and causing contraction of extrinsic tongue muscles has no effect on receptor activity

moses between the hypoglossal and vagus group nerves The facial reflex did not appear until the A spike of the hypoglossal nerve was almost maximal (Fig 3) and thus was evoked from small fibres, probably not C fibres however, but apparently fibres of the same size as those which mediated the reflex response elicited by stimulation of the saphenous nerve, *i e* delta fibres A similar facial reflex could be elicited by pain stimuli to different parts of the head trunk and limbs The reflex is presumably identical with the "movement of the vibrissae" described by Woodworth and Sherrington (1904 and which they considered one of the most discrete and easily elicited components of a nociceptive reaction "the pseudoaffective reflex" in decerebrate cat As Reis (1961) points out, the facial reflex has similarities with the palmomental reflex in man, which he considers represents "a low threshold plateau of a generalized somatic response to nociceptive stimuli" Of the natural stimuli, only pain induced suppression of shivering It is therefore highly possible that the afferent fibres in the hypoglossal nerve which mediate these reactions are "pain fibres"

Rise of blood pressure and dilatation of the pupil are generally taken as conventional indices of pain reactions in experimental animals (Bishop 1946) In chloralosed animals these reactions were elicited by stimulation of the hypoglossal nerve According to Tarkhan (1936) and Downman (1939) this also applies in urethanized cats and cats decerebrated under ether anesthesia We were unable to evoke these reactions in cats anesthetized with pentobarbitone Thus the reactions are not particularly strong which may be due to the fact that the afferent fibres mediating the reactions are few in number

2 *The reflex responses in the styloglossus muscle and inhibition of tonic activity in this muscle* The stimulus threshold for these effects was slightly lower than that required to elicit movement of the vibrissae It is difficult to say whether the reason for this was that the afferent fibres are larger or that it was not necessary to activate an equally large number of fibres to obtain the reflex effects in the styloglossus muscle

Tarkhan (1936) recorded contralateral reflex movements in the tongue elicited by stimulation of the central cut end of the hypoglossal nerve, an observation not confirmed by other workers (see Blom 1960) The hypoglosso-hypoglossal reflex

demonstrated in this study is obviously not a monosynaptic reflex the afferent fibres of the reflex arc have a higher stimulus threshold than the efferent Nothing indicates that it is a myotatic reflex of the kind studied by electrical nerve stimulation in other cat muscles

The inhibition of tonic activity in the styloglossus muscle induced by stimulation of a hypoglossal nerve filament from this muscle was interpreted by Blom (1960), who first described this phenomenon as a recurrent inhibition Accordingly this would indicate the existence of recurrent collaterals in the hypoglossal nucleus although Cajal (1909) was unable to find any If the inhibition were recurrent it should have the same threshold as the motor fibres but its threshold is in fact considerably higher (*cf* also Porter 1965) The fact that intracranial section of the vagus group abolished the inhibition makes it unlikely that it is recurrent

3 That pain stimuli to the tongue evoked no definitely identifiable impulse activity in proximal hypoglossal filaments was probably due to the technical reasons mentioned earlier In a few instances however we succeeded in recording activity elicited by stretching of the tongue (Fig 9 10) Cooper (1954) and Blom (1960) recorded activity in distal hypoglossal filaments close to their exit from the intrinsic muscles on stretching of the tongue On the other hand such activity has not earlier been shown in the proximal parts of the nerve and consequently it has not been possible to determine whether the impulses reach the brain stem via the hypoglossal or the lingual nerve Our results show that such impulses travel in the hypoglossal nerve beyond its connection with the lingual nerve but we do not know by which cranial nerve root they enter the brain stem

The receptors we studied behaved in every way like muscle spindles but can of course have been some other type of stretch receptors The fact that despite long lasting experiments on several cats with recording from several hundred filaments we were only able to record activity in a total of 10—15 such receptors probably indicates that the afferent fibres from them in the hypoglossal nerve are few in number This is also in agreement with the anatomical observations of Blom It has not been possible to demonstrate activity from stretch receptors of this type in the lingual nerve (Blom 1960 *cf* also Porter 1966) Therefore Blom's conclusion that other types of receptors situated in the submucosa rapidly adapting and sensitive among other things to phasic movements in the tongue muscles are of greater importance for movements of the cat's tongue appears to be well founded

Cooper (1953) found muscle spindles in the intrinsic muscles of the tongue in man and rhesus monkey To judge from the investigations of Bowman and Combs (1968) in the rhesus monkey fibres from stretch receptors of the muscle spindle type in the distal half of the hypoglossal nerve are considerably more numerous in these animals than in the cat The authors assume that in the rhesus monkey the muscle spindles are of vital importance for the proprioception of the tongue and mean that there is no reason to ascribe the touch receptors a special role Accordingly there appears to be fundamental differences in the anatomical and functional substra for the proprioception of the tongue in different species of higher mammals

References

BARNARD J W The hypoglossal complex of vertebrates *J comp Neurol* 1940 *72* 489—524

BARRON, D H, A note on the course of the proprioceptor fibres from the tongue *Anat Rec* 1936 *66* 11—15

BISHOP, G H, Neural mechanisms of cutaneous sense *Physiol Rev* 1946 *26* 77—102

BLOM, S, Afferent influences on tongue muscle activity *Acta physiol scand* 1960 *49* Suppl 170

BOWMAN, J P and C M COMBS, Discharge patterns of lingual spindle afferent fibers in the hypoglossal nerve of the rhesus monkey *Exp Neurol* 1968 *21* 105—119

BOYD J D, The sensory component of the hypoglossal nerve in the rabbit *J Anat* (Lond) 1941 *75* 330—345

BRODAL, A Central course of afferent fibers for pain in facial, glossopharyngeal and vagus nerves *Arch Neurol Psychiat* (Chic) 1947 *57* 292—306

CAJAL, S R y, *Histologie du système nerveux de l'homme et des vertebrés* I A Maloine Paris 1909

CLARA M, *Das Nervensystem des Menschen* 3rd edition J A Barth Verlag, Leipzig 1959

COOPER, S, Muscle spindles in the intrinsic muscles of the human tongue *J Physiol* (Lond) 1953 *122* 193—202

COOPER, S, Afferent impulses in the hypoglossal nerve on stretching the cat's tongue *J Physiol* (Lond) 1954 *126* 32 P

CORBIN, K B and F HARRISON, Further studies on tongue innervation *Proc Soc exp Biol* (N Y) 1938 *38* 308—310

DICKINSON, C J, *Electrophysiological Technique* Electronic Engineering (Lond) *1950* 141 p

DOWNMAN, C B B Afferent fibres of the hypoglossal nerve *J Anat* (Lond) 1939 *73* 387—395

FITZGERALD, M J T and M E LAW, The peripheral connexions between the lingual and hypoglossal nerves *J Anat* (Lond) 1958 *92* 178—187

FRORIEP, [illegible] Ganglion, in der R[illegible]

GREEN, [illegible] *Nature* (Lond

GREEN J D and K NEGISHI, Membrane potentials in hypoglossal motoneurons *J Neurophysiol* 1963 *26* 835—856

HINSEY J C and K G CORBIN, Observations on the peripheral course of the sensory fibers in the first four cervical nerves of the cat *J comp Neurol* 1934 *60* 37—44

HOLLIGER A Contribution a l'etude de la constitution de l'anse du nerf hypoglosse, etude d'anatomie comparee *Arch Anat Hist Embr* 1955 *38* 4—46

LANGWORTHY O R A study of the innervation of the tongue musculature with particular reference to the proprioceptive mechanism *J comp Neurol* 1924a *36* 273—297

LANGWORTHY O R Problems of tongue innervation Course of proprioceptive nerve fibers autonomic innervation of skeletal musculature *Bull Johns Hopkins Hosp* 1924b *35* 239—246

LAW, M E Lingual proprioception in pig dog and cat *Nature* (Lond) 1954 *174* 1107—1108

PORTER, R Synaptic potentials in hypoglossal motoneurones *J Physiol* (Lond) 1965 *180* 209—224

PORTER, R Lingual mechanoreceptors activated by muscle twitch *J Physiol* (Lond) 1966 *183* 101—111

REIS, D J The palmomental reflex A fragment of a general nociceptive skin reflex A physiological study in normal man *Arch Neurol* 1961 *4* 486—498

SAUERLAND, E K and N MIZUNO Hypoglossal nerve afferents elicitation of a polysynaptic hypoglosso-laryngeal reflex *Brain Res* 1968 *10* 256—258

SNIDER R S and W T NIEMER *A Stereotaxic Atlas of the Cat Brain* University of Chicago Press, Chicago 1961

TARKHAN, A A Über die Vorhandensein afferenter Fasern im Nervus hypoglossus *Arch Psychiat Nervenkr* 1936 *105* 475—483

TARKHAN, A A and I ABOU EL NAGA Sensory fibres in the hypoglossal nerve *J Anat* (Lond) *1947* *81* 23—32

TARKHAN, A A and S ABD-EL MALEK On the presence of sensory nerve cells on the hypoglossal nerve *J comp Neurol* 1951 *93* 219 228

WOODWORTH R S and C S SHERRINGTON A pseudaffective reflex and its spinal path *J Physiol* (Lond) 1904 *31* 234—243

Acta physiol. scand. 1970. 79. 37—49

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The Stimulating Effect of Angiotensin and Vasopressin on Adenosine Triphosphatase Activity in vitro

By

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Abstract

LEVIN, K. *The stimulating effect of angiotensin and vasopressin on adenosine triphosphatase activity in vitro* Acta physiol scand 1970 79 37—49

The eff... ...ogenates of rat kidney cortex ... of angiotensin per ml of in- ... , K^+, EDTA and imidazole ... was observed The presence of Mg^{++} and Na^+ was found to be necessary for the demonstration of angiotensin stimulation. The optimal pH for the effect of angiotensin was found to be 7 2 and this was confined to very narrow limits. The degree of stimulation was greatest in fresh homogenates and this rapidly decreased on storage Centrifugation of the homogenates indicated that the ATPase activity which could be stimulated by angiotensin was particle bound A similar effect to the one produced by angiontensin was obtained with vasopressin though not with oxytocin or a number of other vasoactive substances known to effect the renal handling of sodium ions Stimulation by angiotensin could be demonstrated in kidney cortex from mouse, guinea pig rabbit, rat and from man, and in homogenates from a number of other tissues It was concluded that the stimulation of ATPase by angiotensin was brought about through the same mechanisms as the stimulation produced by ouabain, and that both these phenomena are closely related to Na^+ K^+ ATPase The possible physiological significance of these findings is discussed

The particle bound, Na and K stimulated adenosine triphosphatase activity (Na-K^+ ATPase) first described by Skou in 1957 and 1960 has been demonstrated to occur in tissues from several organs including the kidney (Whittam and Wheeler 1961, Skou 1962, Jørgensen 1968 and others) There is strong evidence for the view that this enzymic activity is closely related to the active transport of sodium and potassium ions through cellular membranes (Post *et al* 1960) An extensive review covering this field and which emphasizes the transport of sodium ions in the kidney was recently published by Katz and Epstein (1968)

The inhibition of ATPase activity by ouabain and other cardioactive glucosides has been extensively studied (for review see Skou 1965), however much less attention has been paid to the stimulating effect on ATPase activity of ouabain when present in very low concentrations This latter effect of ouabain was first

by Repke (1963). Palmer and Nechay (1964) have later been able to show a correlation between ouabain stimulation of ATPase activity and an increased reabsorption of sodium in chicken kidneys. The conditions necessary for the demonstration *in vitro* of ouabain stimulation of ATPase activity has further been studied by Palmer, Lasseter and Melvin (1966).

The effect of angiotensin on the control of sodium flux in the kidney has been studied in various experimental animals and in man (Cannon, Ames and Laragh 1966, Barraclough, Jones and Marsden 1967, Jahn *et al.* 1967). Besides the well known effects on the hemodynamics and release of aldosterone it has regularly been found that angiotensin in small doses decreases natriuresis and that higher doses exert the reverse effect. As to whether these effects arise from the direct action of angiotensin on the reabsorption of sodium ions by the renal tubuli or in point of fact are secondary to changes in the renal hemodynamics is a controversial point. Leyssac (1968 and 1965) proposed that angiotensin promoted natriuresis by blocking reabsorption in the proximal tubuli.

This situation led to the present study in which the effects of angiotensin on ATPase activity *in vitro* were examined. In an earlier investigation by Bonting, Canady and Hawkins (1964) no such effects could be demonstrated.

Methods

Assay animals. Male Sprague Dawley rats (150 to 250 g) were given a mixed diet which included a pelleted rat feed containing ample amounts of vitamins together with drinking water *ad libitum*. Other species of animals were reared under similar conditions.

Materials. Adenosine triphosphate disodium salt (ATP) and adenosine diphosphate sodium salt (ADP): Sigma Chemical Co., St. Louis, Mo., USA or C. F. Boehringer & Soehne GmbH, Mannheim, Germany.

Synthetic Asp-β-amide of val-5-angiotensin II lyophilized with 20 parts per weight of mannitol (Hypertensin N, Ciba, Basel, Switzerland).

Synthetic vasopressin and oxytocin by courtesy of AB Ferring, Malmö, Sweden.

Ethylenediaminotetra-acetic acid, di-Na-salt (EDTA): Hopkin and Williams, Essex, Great Britain.

Other chemicals were *pro analysi* preparations and ordinary pharmaceutical preparations of hormones and therapeutic agents.

Preparations of homogenates. The animals were killed by exsanguination which was achieved by cutting the carotid arteries open after the animals had been rendered senseless by a blow on the back of the head. One kidney was removed and a piece of the cortex cut out. This was then weighed and immediately transferred to an all-glass Potter-Elvehjem homogenizer containing either 0.25 M sucrose or 0.25 M sucrose together with 5 mM EDTA and 25 mM imidazole pH 6.8 cooled to 0° C. The samples were then homogenized. In those experiments where tissues from other organs were examined a similar procedure was adopted.

Determination of ATPase activity. Apart from those cases noted in the text, determinations were performed in a medium containing 2 mM Mg, 78 mM Na, 5 mM K, 77 mM Cl, 25 mM imidazole, 0.13 mM EDTA and 3 mM ATP adjusted to pH 7.2. Angiotensin was then added at a concentration of 1.7 μg/ml (1.6 μM) and ouabain at a concentration of 0.6 mg/ml (0.8 mM).

Two methods of determination were used with equal success. In the first case 20 μl of a 0.3 to 0.6 % homogenate were added to 200 μl of the reagent in small test tubes and in the second case 20 μl of a 3 to 6 % homogenate were added to 2 ml of the reagent in small bottles. In both cases the reagent was warmed to 37° C prior to the addition of the homogenate. The homogenates were added within 10 min after sacrifice of the animal. The samples were in

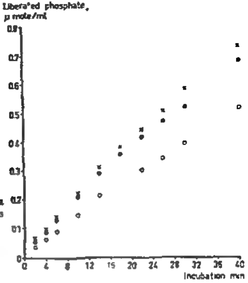

Fig 1 Phosphate liberation from ATP by a kidney cortex homogenate For the conditions of incubation, cf Methods

The symbols represent the various media
● = basal medium
○ = with 0.8 mM ouabain
× = with 1.6 μM angiotensin

cubated at 37° C for 20 min and the reactions were stopped by the addition of 10 % trichloroacetic acid (TCA) in the ratio of 1 vol ... of TCA ...
After centrifugation
method of Sumner
When different bu
appropriate solutions In the absence of ATP, or when TCA was added prior to the addition of the homogenate, no increase in the phosphate reading was noted Incubations were performed in duplicate and the standard deviation between samples was found to be less than 2 % When variations between the blanks were included the standard deviation for duplicate determinations was 2.8 % Protein determinations were made using a modification of the procedure of Lowry (Holmgård 1964, p 22)

ATPase activity in the basal medium i.e. without further additions was calculated and expressed in millimoles of phosphate liberated per gram protein per hour mmoles Pi/g prot./hr) This was termed the basal ATPase activity Any changes in the rate of phosphate liberated registered on the addition of other substances were calculated as a percentage of this basal activity Values are given as M ± S.E.M

Results

Preliminary experiments showed that an increase in the release of inorganic phosphate occurred upon the addition of angiotensin to the incubation medium. In Fig 1 are shown the effects of angiotensin and ouabain when added to the incubation medium. In all three cases (control + angiotensin + ouabain), the buffer compositions were the same and the samples of homogenate derived from a common stock It can be seen that angiotensin stimulated the release of inorganic phosphate and that the degree of stimulation was constant over the period of time in which the incubation was followed Ouabain on the other hand inhibited phosphate release an effect found in previous investigations At no instance was more than 20 % of the substrate consumed as calculated from the results Since the graphs obtained were almost linear for up to 20 min this period of time was used in further experiments

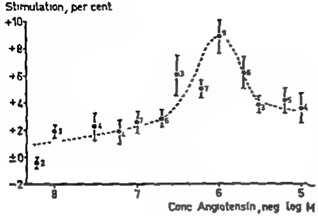

Fig 2 Stimulation of basal ATPase activity with different concentrations of angiotensin in the incubation medium Each point represents the mean value of a number of experiments indicated The vertical bars represent the standard error of mean

In a group of 11 rats the mean value of the increase in ATPase activity following the addition of angiotensin at a concentration of 1 7 μg/ml was 8 6±0 5 % The basal ATPase activity was 19 2±0 4 mmole Pi/g prot /hr

The results obtained in the above 11 expts on the effect of angiotensin on tissue homogenates from rats were fairly consistent, however in other experiments considerable divergences from the mean value were obtained, even when the conditions of assay were the same In approximately 5 % of the experiments no effect of angiotensin was obtained, whereas in others as much as 20 % stimulation was found

4) The effect of variations in the incubation media

a) Angiotensin

In several experiments the concentration of angiotensin in the basal medium was varied The results of these experiments are presented in Fig 2 In all experiments the optimal stimulation occurred between 0 3 to 2 0 μg of angiotensin per ml of incubation medium Because of the presence of mannitol in the preparation, which is known to interfere with the phosphate determinations, concentrations on angiotensin above 10 μg/ml could not be used

In further experiments the angiotensin concentration in the incubation medium was investigated in order to determine to what extent it was influenced by the action of angiotensinase present in the homogenates Angiotensin was assayed by the method of Gunnels *et al* (1967) by following the effect of a small sample of medium on the blood pressure of a rat Samples of the incubation medium without ATP were first diluted 1 10 with saline then injected intravenously into a nephrectomized rat It was found that after 20 min of incubation at 37° C more than 50 % of the added angiotensin still remained in the medium

b) pH

In 5 experiments the pH of the medium was varied by either the addition of NaOH or HCl (Fig 3) The optimal pH for the stimulation of ATPase by angiotensin was

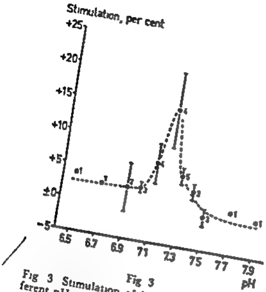

Fig 3

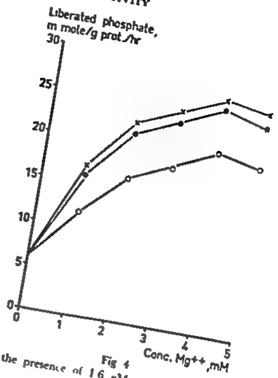

Fig 4

Fig 3 Stimulation of basal ATPase activity in the presence of 1 6 μM angiotensin at different pH Symbols as in Fig 2

Fig 4 ATPase activities upon incubation with different Mg concentrations Other experimental conditions and symbols as in Fig 1

found to be 7 2 with a rapid declination towards higher and lower values The pH of the medium was constant during the period of incubation

c) Cations

In Fig 4 is shown the effect of varying the magnesium ion concentration in the incubation medium Although the basal ATPase activity was greatly influenced by a variation in the magnesium ion concentration between the limits of 1 to 5 mM the relative stimulation obtained with angiotensin was not significantly altered In the total absence of magnesium ions the basal ATPase activity was one third the activity obtained in the presence of 1 mM Mg and under these conditions no stimulation was obtained with angiotensin

The effect of Ca^{++} present in concentrations from 0 2 mM to 1 mM was also investigated In these experiments EDTA was excluded from the medium (Table I) The results showed considerable variation in the basal ATPase activity It was found that the degree of inhibition with ouabain decreased, and in cases disappeared, with increasing Ca^{++} concentrations The results obtained with angiotensin also showed some variance, the degree of stimulation being very low and appearing independent of the calcium ion concentration In the absence of calcium the percentage stimulation with angiotensin was $2\,1 \pm 0\,8$ % It is possible that the relatively small effect

TABLE 1 The effect of increasing Ca^{++} concentrations in the incubation medium The results are expressed in percentage of the basal ATPase activity ±S E M

Concentration of Ca^{++} mM	Number of experiments	Stimulation with angiotensin %	Inhibition with ouabain %
0	6	2 1±0 8	26 7±2 6
0 2	6	3 7±0 7	9 3±3 5
0 4	6	1 9±0 6	5 6±2 5
0 6	6	0 2±2 8	[illegible] 3±2 5
0 8	5	2 0±0 6	3 8±1.5
1 0	6	1 7±0 7	6 0±2 9

obtained with angiotensin in these experiments was due to the exclusion of EDTA from the medium rather than to variations in the calcium ion concentration The residual calcium ion concentration due to the presence of trace amounts in the homogenate was calculated to be below 1 5 μM

In the absence of sodium ions no stimulation with angiotensin was observed whereas in the absence of potassium ions angiotensin stimulation was normal

d) ATP and ADP

In Fig 5 are shown the results of an experiment in which the levels of ATP were ied A stimulating effect due to angiotensin was noted to occur when ATP was present at concentrations between 1 5 to 5 0 mM, and in further experiments it was shown that the degree of stimulation decreased with increasing concentrations of ATP

Different commercial ATP preparations gave similar results although the blank values obtained in the phosphate determinations varied considerably

Fig 6 shows the effect of increasing concentrations of ADP in the incubation medium It can be seen that increasing concentrations of ADP lower the basal ATPase activity and decrease the relative inhibition obtained with ouabain and the degree of stimulation obtained with angiotensin When 4 mM ADP was substituted for the ATP in the incubation medium a phosphate release of 6 2 m mole Pi/g prot /h was obtained The addition of 1 7 μg of angiotensin per ml did not influence the result whereas 0 6 mg of ouabain per ml caused a 9 % inhibition in the rate of phosphate liberation

When ATP in the incubation buffer was exchanged for 0 8 mM phosphate there was no change in phosphate concentration after incubation

The addition of 0 7 mM phosphate to the normal incubation medium did not affect the angiotensin stimulation of phopshate release

2 *Different preparations of homogenate*

On keeping the homogenates in an icebath for 2 hrs prior to use, a decrease of up to 20 % occurred in the basal ATPase activity and the stimulatory effect of angio-

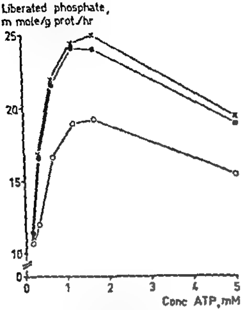

Fig 5

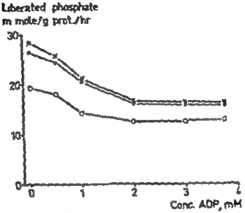

Fig 6

Fig 5 ATPase activities upon incubation with different ATP concentrations Other experimental conditions and symbols as in Fig 1

Fig 6 ATPase activities obtained upon addition of increasing concentrations of ADP to the basal incubation medium Other conditions and symbols as in Fig 1

tensin was very much lower After 1 hour of storage the effect of angiotensin was greatly decreased (Fig 7) The stability of homogenates tended to be greater when they were stored at higher concentrations In experiments where homogenates were lyophilized immediately after preparation angiotensin still showed a stimulatory effect after 24 hrs though this was less than one half the original effect In these samples the basal ATPase activity was only slightly lower Freezing and thawing of a homogenate had a similar effect

During the course of the investigation the effect of the medium used in the preparation of the homogenates was examined Besides 0 25 M sucrose 0 25 M and 0 05 M glucose the normal incubation medium minus the ATP and distilled water were tested No differences occurred in the results obtained from these different systems The addition of 0 2 % deoxycholate to 0 25 M sucrose resulted in a somewhat lower basal ATPase activity than when sucrose alone was used However there was no decrease in the degree of stimulation obtained with angiotensin The addition of 5 mM EDTA and 25 mM imidazole pH 6 8 to 0 25 M sucrose resulted in more constant determinations than when sucrose was used alone

3 *Fractionation of the ATPase system*

Centrifugations were performed in a small laboratory centrifuge (Misco Microcentrifuge) which at its highest speed produced a centrifugal force of 29 000 × *g* The

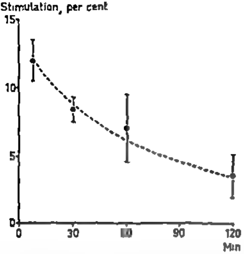

Fig 7

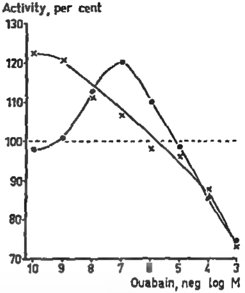

Fig 8

Fig 7 The effect of aging of homogenates upon the angiotensin stimulation of ATPase
Mean values and S E M of 6 expts where the homogenates have been kept in an ice bath
Symbols as in Fig 2

Fig 8 The effect upon the basal ATPase activity of different concentrations of ouabain
● = without angiotensin
× = in the presence of 1 6 μM angiotensin

temperature in the sample tubes rose to 10° C during centrifugation In these experiments rat kidney cortex homogenized in 0 25 M sucrose was used

After an initial centrifugation at 5 000 $\times g$ for 10 min the supernatant was decanted and recentrifuged at 29 000 $\times g$ The pellets obtained by centrifugation were resuspended in 0 25 M sucrose In the first pellet only a small part of the activity stimulated with angiotensin was detected and the major part was found in the second pellet and in the slightly opalescent supernatant This opalescence was most probably due to the occurrence of light microsomes in this fraction The partition of the ATPase activities between the 29,000 $\times g$ pellet and the supernatant varied but a large part of it was always found in the pellet In some cases almost all the activity stimulated by angiotensin was found in the 29,000 $\times g$ pellet

4 *Effect of other substances*

a) Ouabain

Using the same enzyme preparations and techniques outlined above it was confirmed that ouabain when present in low concentrations stimulated ATPase activity (Fig 8) In the presence of 1 7 μM of angiotensin increasing concentrations of ouabain resulted in lower levels of ATPase activity It would thus appear that angiotensin and ouabain act antagonistically towards each other These results are dealt with in the discussion

It was subsequently shown that in experiments where homogenates were kept in

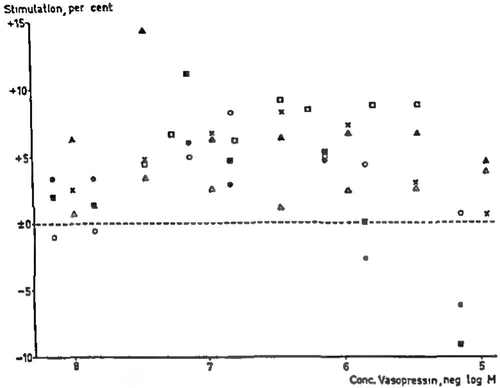

Fig 9 Stimulation of ATPase activity with different concentrations of vasopressin 6 expts, each result marked with a different symbol

an icebath for some time the ability of ouabain and angiotensin to stimulate ATPase activity decreased simultaneously

b) Vasopressin

Vasopressin was found to have an effect very similar to the one of angiotensin (Fig 9) The results obtained in the experiments conducted in the presence of different concentrations of vasopressin were more irregular and did not show such a definite optimum as was found with angiotensin

c) Other hormones

Other polypeptide hormones tested were oxytocin present at 10 and 20 I U /ml and secretin from 0 02 to 2 clinical units/ml of incubation medium However, these were without any effect on the basal ATPase activity Adrenaline, 7 and 13 μg/ml, noradrenaline, histamine and L-thyroxine present at 20 and 40 μg/ml, and aldosterone 2 to 20 μg/ml were similarly without effect

It was found that noradrenaline 5 μg/ml in combination with angiotensin from 1 7 ng/ml to 1 7 μg/ml and aldosterone from 2 to 20 μg/ml in combination with 1 7 μg/ml of angiotensin were ineffective the only effect on ATPase activity in these cases being the expected one due to the presence of angiotensin

TABLE II Mean values in percentage of ATPase stimulation obtained with 1.6 μM angiotensin in homogenates from different tissues

Animal	Kidney cortex		Kidney medulla		Heart muscle		Skeletal muscle		Liver		Adrenal gland	
	n	%	n	%	n	%	n	%	n	%	n	%
Mouse	1	1.5	1	0	1	12.4	1	0	1	0		
Guinea pig	1	13.0	1	[illegible]	1	7	1	0	1	0		
Rabbit	[illegible]	3.0	5	2.1								
Rat	10	8.4	10	3.0	2	3.2					1	0

5. *Effects on other tissues*

a) Animals

In all the experiments described up to now homogenates of rat kidney cortex were used since it was found to be comparatively easy to get uniform preparations from these tissues. However, homogenates from other tissues were also examined. In preparations of kidney medulla the basal ATPase activity was found to be slightly higher than in the cortex and the ouabain inhibited portion was significantly higher, though the relative stimulation obtained with angiotensin was lower (Table II). This difference in preparations from kidney cortex and medulla was found in all the different species of animals examined.

Angiotensin stimulation was regularly found in preparations from heart muscle though not in skeletal muscle, liver and adrenal gland. In some cases homogenates of aortic wall from rat showed a pronounced ATPase stimulation with angiotensin, however this was not always reproducible.

b) Humans

Biopsy samples were taken from macroscopically healthy parts of the kidney cortex during operations on patients at the hospital. Preparations from this material were found to show a stimulation with angiotensin (Table III).

Discussion

Bonting *et al.* (1964) reported that they were unable to show any effect of angiotensin on Na-K ATPase activity in homogenates of rabbit kidney cortex and

TABLE III ATPase activities in human kidney cortex

Case	Liberated Pi μmoles/g prot./hr	Stimulation with angiotensin %	Inhibition with ouabain %
1	6.2	4.1	19.6
2	5.4	4.4	13.2

medulla However it was noted that a slight increase in Mg ATPase activity occurred in some of their experiments In the present investigation it was found that homogenates of rabbit kidney cortex and kidney medulla contain comparatively little ATPase activity which can be stimulated with angiotensin Furthermore in the experiments of Bonting *et al*, incubations were performed at a pH of 7 5 which deviates significantly from the optimum pH of 7 2 (Fig 3) The choice of tissue and differences in technique employed would probably therefore account for the discrepancy between the present results and those of Bonting *et al*

The techniques used in the preparation of tissues and in the assay of ATPase activity of the homogenates were chosen in order to obtain the greatest effect with angiotensin with acceptable reproducibility These were not necessarily the optimum conditions for the basal ATPase activity In those experiments where it was attempted to separate the ATPase activity stimulated with angiotensin from other forms of ATPase activity no increase in the percentage stimulation was observed However this could well have been due to aging of the sample during preparation

The ATP concentration used in the incubation medium was in excess of the optimal concentration A further increase would therefore have resulted in a decrease in the rate of phosphate liberation. This is evident from Fig 5 Consequently the mechanisms by which angiotensin is able to stimulate the rate of phosphate liberation could be

1 To an effective lowering in the ATP concentration This could be brought about by either

a) a direct combination of angiotensin to ATP or

b) by an inhibition of ATP resynthesis Since the molar concentration of angiotensin was generally some 2500 times lower than that of ATP it would seem unlikely that a direct binding to ATP would bring about the observed effect When ADP was used as the substrate it was evident that considerable amounts of adenylate kinase were present in the homogenates However angiotensin was without effect in these cases which seems to exclude the possibility that angiotensin inhibits ATP resynthesis

2 To a physical effect To date no evidence has been reported for such an effect However the possibility cannot be excluded

3 To an inhibition of some reaction which may utilize inorganic phosphate liberated from ATP by the action of ATPase This possibility is ruled out by the experiments performed in phosphate medium where no consumption of phosphate could be demonstrated

4 To a stimulation of ATPase activity brought about by a direct interaction of angiotensin with the enzyme system itself

The pH optimum for the stimulation of ATPase by angiotensin was found to be 7 2 with very narrow limits this is in contrast to what is known about Na K-

ATPase, where the corresponding value is about 7 5 with a much broader maximum (Kingsolving Post and Beaver 1963)

When homogenates were centrifuged and the various fractions assayed for angiotensin sensitive ATPase activity, the majority of the activity was recovered from where one would expect the main part of the microsome fraction to occur These findings would therefore seem to indicate that ATPase of this type is normally associated with these particles

Examination of the effect of angiotensin in the presence of varying concentrations of ouabain (Fig 8) indicate that the effects of these substances are somehow interrelated In the presence of ouabain the stimulatory effect of angiotensin was lowered and in the presence of high concentrations the effect was completely inhibited There is probably a mutual competition between angiotensin and ouabain as for the stimulation of ATPase The finding that the angiotensin stimulated ATPase is at least partially particle bound, that it is dependent on the presence of Mg^{++} and Na^{+}, and that it can be inhibited by ouabain indicates a close relationship of this enzyme fraction to Na^{+}-K^{+} ATPase, and consequently to the active transport of ions through the cellular membranes

The considerable variation in the results obtained for the effect of angiotensin on homogenates from different rats could not be explained solely on the grounds of methodical error and is therefore presumed to have been due to individual differences These variations may be explained in several ways and in a following paper (Levin 1970) the influence of such factors as the age of the animal, the endogenous angiotensin supply and stress will be considered

Cannon *et al* (1966) Jahn *et al* (1967), and Barraclough *et al* (1967) have reported that angiotensin when given in small doses had a stimulatory effect on renal tubular sodium reabsorption Similarly, Turker, Page and Khairallah (1967) found that angiotensin stimulates sodium efflux from smooth muscle These latter authors suggested that the effects could be due to a stimulation of membrane ATPase The results of the experiments presented here, although performed *in vitro* might support this hypothesis

The concentrations of angiotensin found to be necessary for the stimulation of ATPase *in vitro* were far in excess of those required for biological effects However it seems probable that angiotensin is not homogeneously distributed throughout the tissues and that the concentration may be increased at the effector site This view is supported by the finding that the concentration of angiotensin in venous blood is considerably lower than that in arterial blood when a constant infusion of angiotensin is given The difference in concentrations is much greater than what can be accounted for by the action of angiotensinase (Boucher *et al* 1964) Furthermore the concentration of angiotensin has been found to be considerably higher in kidney tissue than in blood following infusion (Bumpus *et al* 1964)

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References

Boucher, R, R Veyrat, J de Champlain and J Genest, New procedures for measurement of human plasma angiotensins and renin activity levels *Canad med Ass J* 1964 *90* 194—201

Bumpus, F M, R R Smeby, I H Page and P A Khairallah, Distribution and metabolic ... 1964 *90* 190—193

C ... angiotensin infusion to in- ... 1966 *211* 1021—1030

G ... ..., Plasma renin activity ... 1967 *119* 232—240

Holmoård, A., Quantitative analysis of enzymes in normal and diseased kidney tissue *Scand J clin Lab Invest* 1962 *14* Suppl 65

Jahn, H, P Reville, T Kurtz, F Kuntzmann and J Stahl Quelques aspects de l'action ... —648

... ATP hydrolyzing enzyme system in ... of sodium on the enzyme system

... in potassium-activated adenosintriphosphatase in the transport of cations across biological membranes *New Engl J Med* 1968 *278* 253—261

Kinsolving, C R., R L. Post and D L Beaver, Sodium + potassium transport and adenosine triphosphatase activity in kidney *J cell comp Physiol* 1963 *62* 85—93

... lation *in vitro* of rat kidney

... tubular reabsorption of salt

Le ... the proximal tubula ... *64* 167—175

Pa ... Na⁺ and K⁺ dependent ... —633

Palmer, R F, and B R Nechay, Biphasic renal effects of ouabain in the chicken Correlation with a microsomal Na^+-K^+ stimulated ATPase *J Pharmacol exp Ther* 1964 *146* 92—98

Post, R L, C R Merritt, C R Kinsolving and C D Albright, membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte *J biol Chem* 1960 *235* 1796—1802

Repke, K, Influence of cardioactive principles on pump ATPase *Proc of I int Pharmacol Meet August 22—25, 1961* Ed W Wilbrandt Pergamon Press, Oxford 1963 *3* 65—73

Skou, J C, The influence of some cations on an adenosine triphosphatase from peripheral nerves *Biochim biophys Acta* (Amst) 1957 *23* 394—401

Skou, J C, Further investigations on a $Mg^{++}+Na^+$-activated adenosintriphosphatase possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane *Biochim biophys Acta* (Amst) 1960 *42* 6—23

Skou, J C, Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na^+ and K^+ *Biochim biophys Acta* (Amst) 1962 *58* 314—325

Skou, J C, Enzymatic basis for active transport of Na^+ and K^+ across cell membrane *Physiol Rev* 1965 *45* 596—617

Sumner, J B, A method for the colorimetric determination of phosphorus *Science* 1944 *100* 413—414

Turker, R. K., I H Page and P A Khairallah Angiotensin alteration of sodium fluxes in smooth muscle *Arch int Pharmacodyn* 1967 *165* 394—404

Whittam, R and K P Wheeler, The sensitivity of a kidney ATPase to ouabain and to sodium and potassium *Biochim biophys Acta* (Amst) 1961 *51* 622—624

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Influence of Stress, Adrenalectomy and Age on the Stimulation in vitro of Rat Kidney ATPase by Angiotensin

By

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Abstract

LEVIN, K. *Influence of stress, adrenalectomy and age on the stimulation in vitro of rat kidney ATPase by angiotensin.* Acta physiol. scand. 1970. 79. 50—57.

The previously demonstrated stimulation of kidney ATPase by angiotensin was studied with respect to variations due to stress, anesthesia, noradrenaline activity, adrenal function and age of the animals. The kidney cortex ATPase from rats subjected to stress, pain or angiotensin injection could not be activated *in vitro* by angiotensin. This was considered to be due to a maximal stimulation of the enzyme already at sacrifice of the animals. The susceptibility of the enzyme to angiotensin stimulation disappeared after adrenalectomy, but remained if the animals were treated with aldosterone. Angiotensin had no stimulating effect on the kidney cortex ATPase in newborn rats; the effect reached a maximum when the animals weighed 150—200 g and again disappeared when the animals grew larger.

In an earlier paper (Levin 1970) the stimulation of rat kidney adenosine triphosphatase (ATPase) by angiotensin, *in vitro,* was reported. The experiments described in this paper were undertaken in order to investigate the influence of the condition of the assay animal upon this stimulation, to find a possible explanation for the irregularity in the stimulation and to see if the angiotensin stimulation of ATPase *in vitro* could be correlated to the effect of angiotensin upon kidney function *in vivo*.

The susceptibility, *in vitro,* of kidney ATPase to angiotensin may be influenced by the condition of the animal at the time of sacrifice. For this reason rats were subjected to psychic and physical stress, anesthesia, and i.v. angiotensin injection before the kidneys were examined for angiotensin stimulation. The effects of adrenalectomy, aldosterone treatment and age of the animals were also studied.

Methods

Where not otherwise noted, male Sprague-Dawley rats 150—250 g were used. The animals were killed and homogenates of the kidney cortex were prepared and assayed for ATPase activity as described previously (Levin 1970).

Alkaline phosphatase was determined according to Lowry *et al.* (1954).

Bilateral adrenalectomy was performed during ether anesthesia by incision from the dorsal side. After the operation the animals were kept in ordinary room temperature with free admittance to food and tap-water, as were the control animals. No extra sodium was given. The

animals showed no signs of deterioration in their general condition during the first 10 days after the operation.

In a second series of experiments anesthesia was started with Halothane and continued with ether. The left kidney was excised following laparotomy, a homogenate of the cortex was prepared and incubated with ATP 5 min following the excision of the first kidney. 0.5 ml of test solution was injected into the abdominal caval vein by a syringe with a fine hypodermic cannula. The test solutions were 0.9 % NaCl, angiotensin 500 and 50 ng per ml in saline, and noradrenaline bitartrate 50 and 5 µg per ml in saline. One min after the injection, the right kidney was excised and treated in the same way as the left kidney.

Other chemicals and reagents were analytically pure standard preparations.

Results

Influence of stress

During the course of this study the normal way of sacrificing the animals has been to render them unconscious by a blow to the back of the head, followed by exsanguination by cutting open the carotid arteries with a pair of scissors. It was noted that when the rats were killed quickly by an experienced animal caretaker, there was generally a higher degree of angiotensin stimulation of the ATPase than if the animals were subjected to a more prolonged agony before death.

Two rats were injected with 10 % NaCl solution in the muscles of the hind limbs and were forced to run for 5 minutes before sacrifice. In these animals no stimulation of ATPase with angiotensin could be detected, whereas in the two untreated littermates investigated at the same time, there was 3.4 and 6.3 per cent stimulation.

Influence of anesthesia

Rats were anesthetized by introducing them into a glass jar filled with diethylether fumes and the anesthesia was continued with ether on a cotton pad. No differences were found between the basal ATPase activities in the kidneys of these rats as compared to those obtained after killing the animals in the usual way. The angiotensin stimulation was, however, lower than normally found, M ± S.E.M. 3.9 ± 1.0 per cent, $n = 7$, which is statistically significant ($p < 0.001$) compared with a group of untreated rats.

In another series of experiments, rats were placed in a glass jar containing cotton wool for several hours. Anesthesia could then be commenced without upsetting the animal by spraying Halothane on the cotton wool when the rat was asleep. The anesthesia was then continued with ether. The degree of ATPase stimulation with angiotensin on kidney cortex preparations taken from these animals was the same as that found after killing the rats in the usual way by exsanguination.

Effects of angiotensin and noradrenaline in vivo

The results of experiments with *in vivo* injection of angiotensin and noradrenaline are shown in Fig. 1. When noradrenaline or a high dose of angiotensin was injected, a

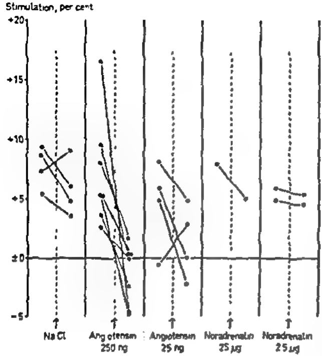

Fig. 1. Percentage stimulation of rat kidney ATPase by angiotensin after intravenous (intracaval) injection of NaCl, angiotensin or noradrenalin. The left dots in each column represent values before infusion (one kidney removed before injection) and the right dots the stimulation after injection (contralateral kidney).

typical blanching of the kidney was observed within 10 sec. A lower dose of angiotensin did not produce this effect, neither did, of course, saline.

Fig. 1 shows that the treatment of the rats with anesthesia and injection of saline did not change the response to angiotensin *in vitro* to any significant degree. It also shows that injection of 250 ng of angiotensin regularly rendered the ATPase insensitive to subsequent stimulation with angiotensin *in vitro*. There is even a slight, although not significant tendency that angiotensin *in vitro* inhibits the basal ATPase after this treatment. 25 ng of angiotensin had an irregular effect.

Noradrenaline, even in large doses, did not produce a comparable effect on the angiotensin stimulation of ATPase activity.

Effect of adrenalectomy

Following adrenalectomy a gradual decrease in the degree of stimulation of ATPase with angiotensin was observed. One week after adrenalectomy no significant stimulation could be found. Preliminary experiments demonstrated that the susceptibility to angiotensin stimulation could be preserved in adrenalectomized rats by i.p. injection of 0.25 mg of aldosterone once a day, provided the last dose was given a few hours before sacrifice. Substitution with hydrocortison did not have this effect.

Table I shows the results from three groups of rats. Group I is a normal control group, identical with that reported in an earlier paper (Levin 1970). These animals were examined during the same period of time as the other groups. Group II con-

TABLE I. ATPase activities in normal kidney cortex homogenates and after adrenalectomy. Activities given as mean values ± standard deviation in mmoles liberated phosphate/g protein/hr. n = number of determinations

Group	Treatment	Activity in medium A	Activity in medium B	Activity in B—A	Activity in medium C	Activity A—C
I	Untreated rats	19.2 ± 1.2 n = 11	20.8 ± 1.4 n = 11	1.7 ± 0.4	14.6 ± 1.4 n = 10	4.7 ± 1.8***
II	Rats 7—10 days after adrenalectomy	21.1 ± 3.2 n = 6	21.4 ± 3.2 n = 5	0.3 ± 0.2*	16.7 ± 2.7 n = [illegible]	4.5 ± 0.6***
III	Rats as in group II, treated with aldosterone 0.25 mg/day i.p.	20.5 ± 3.8 n = 9	21.7 ± 4.1 n = 8	1.2 ± 0.4**	15.9 ± 2.5 n = 9	4.7 ± 1.9***

* significantly different from I, $p<0.001$

** significantly different from I, $p<0.01$, and significantly different from II, $p<0.001$

*** no significant differences between groups

sists of rats adrenalectomized 7—10 days before sacrifice. The group III animals were adrenalectomized in the same way but treated with aldosterone as described above.

In the homogenate of kidney cortex from the animals in group II angiotensin did not stimulate the ATPase activity, whereas a typical stimulation was found in group III. This stimulation was, however, lower than in the control group. The differences between the results in all three groups are significant on the 0.1 % level.

The influence of ouabain on the ATPase activity was also studied in these preparations. There were no significant differences between the groups with respect to the basal ATPase activities or in the degree of inhibition by ouabain. On the other hand, the decrease in angiotensin stimulation was parallelled by a similar decrease in ability to be stimulated by ouabain.

Influence of age

During the course of this investigation it was noticed that there was a tendency for ATPase activity to decrease if the rats grew bigger than about 250 g. The influence of age was subsequently studied.

Two litters of newborn rats were followed from their birth by successive sacrifices at about one week's interval until they had gained weight up to about 100 g. The kidneys from rats less than 50 g were homogenized in one piece and this homogenate was used for the ATPase analysis. The results are shown in Fig. 2 and 3. The results obtained from rats of more than 100 g of weight were from rats purchased at this size. The comparison was considered permissible, since it had been found (Levin 1970) that the difference between cortex and medulla is small with respect to basal ATPase activity as well as to the degree of stimulation.

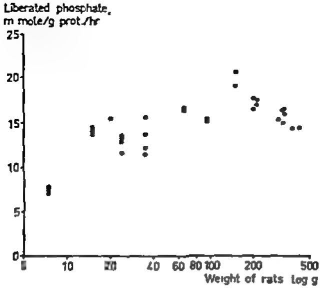

Fig 2 The basal ATPase activity in kidneys from rats of varying weight

Fig 2 illustrates that the basal ATPase activity was low at birth and gradually increased with the age of the rats. This activity tended to be highest in the kidneys from rats of a weight between 150 and 200 g, with a slight decrease with increasing weight and age. The degree of stimulation of the ATPase activity by angiotensin showed the same general pattern (Fig 3) with the exception that practically no stimulation could be observed in the old rats. Since the stimulation is calculated in per cent of the basal ATPase activity the absolute increase of this activity is higher than the increase of basal activity. The per cent of ATPase inhibited by ouabain showed no evident changes with size of the animals. This would indicate that there was a lower Na-K ATPase activity in the young rats. As a comparison, alkaline phosphatase activity was determined in the homogenates from the young rats and related to the protein content. There was no change with increasing age in this activity.

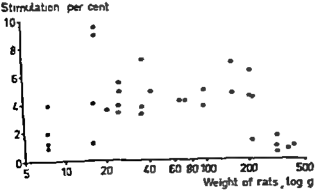

Fig 3 Percentage angiotensin stimulation of the ATPase activities depicted in Fig 2 related to the weight of the animals

The protein content of the kidneys was 10 % of the wet weight in the newborn rats but increased to about 15 % in the adult rats.

In contrast to the results from the experiments described in Fig. 3 where the rats were killed in the common way, an angiotensin stimulation of 3.3 and 5.3 % was found in two 400 g rats killed by an overdose of Halothane, alhough the basal ATPase activity was somewhat lower than the average from 150—250 g rats.

Discussion

A reduction in the degree of ATPase activity stimulated by angiotensin in rats subjected to stress before removal of the kidney was confirmed in several ways. The experiments with intramuscular injection of hypertonic saline immediately before sacrifice cleary demonstrate the influence of pain and scare. The fact that the degree of stimulation was higher when the anesthesia was commenced with Halothane than when ether was used from the start of anesthesia can be explained as a result of diethylether being a sympaticommethic agent, whereas Halothan is known to be parasympaticommethic.

The finding in older rats that stimulation could not be shown when the animals were killed in the conventional way, but was found if they were killed with Halothane can be explained by difficulties experienced in inducing a quick death with rats of increasing size.

It seems probable that the decreased ATPase stimulation in animals subjected to stress and pain is due to an increased endogenous level of the angiotensin content in the kidneys. An increased renin concentration in the blood of humans and rats subjected to work and stress has been demonstrated (Bozovic and Castenfors 1967, Castenfors 1967). It has also been found (Bunag, Page and McCubbin 1966 and others) that noradrenalin and stimulation of sympathetic nerves cause increased renin release from the kidneys. Since stress and pain induce an increased release of catecholamines and stimulate the sympathetic nerves, this could probably lead to an increased angiotensin content of the kidney preventing a further stimulation by addition of angiotensin *in vitro*. The observation that the kidney ATPase could not be stimulated by angiotensin if this hormone had been injected into the animals before sacrifice is a further support of the hypothesis that the susceptibility to further stimulation disappears if the kidneys are already loaded with this hormone.

An increased release of renin and an augmentation of the renin content in the kidneys after adrenalectomy have been observed in the rat by Sokaba *et al.* (1963).

It may be permissible to conclude that an increased release of renin in the rats included in this study resulted in a considerable local production of angiotensin in the kidneys, precluding any further stimulation of the ATPase activity of angiotensin added *in vitro* to the tissue homogenate.

The present results demonstrate that adrenalectomy does not significantly depress the ouabain inhibited ATPase activity in crude homogenates of the rat kidney cortex, whereas the ability of the enzyme to be stimulated by ouabain in low

disappears after adrenalectomy, as does the susceptibility to angiotensin stimulation Persistence after adrenalectomy of the ouabain inhibited ATPase in crude kidney homogenates has been previously observed (Jorgensen 1967) This persistence may be compared with the reduction of the ouabain inhibited activity observed in microsomal ATPase fractions obtained by ultracentrifugation and by further treatment by dialysis (Landon Jazab and Forte 1966) or deoxycholate (Chignell and Titus 1966, Jorgensen 1967) It seems probable that the difference between the crude homogenate and the treated preparation in this respect is not only a change in the enzyme pattern but that it may also be due to changes in membrane structure and localization of the ATPase activity

The failure to demonstrate the stimulation following adrenalectomy may be compared with the observation of Peters (1964) that the natriuretic response in rats to an i v injection of angiotensin disappears within one week after adrenalectomy and that this response can be partly restored by aldosterone administration The time course of the disappearing of natriuretic response to angiotensin is concomitant to the disappearing of the ability of angiotensin to stimulate ATPase activity This seems to indicate that the stimulation of the transport ATPase can be a physiological activity of angiotensin

The absence of a stimulating effect of angiotensin on the ATPase from kidneys of newborn and very young rats and rabbits may be due to the fact that the kidney function is not fully developed at birth (Barnett and Vesterdal 1953) In support of this it has been demonstrated that the tubular concentration ability in children does not reach the adult level until the age of 3 to 6 months (Winberg 1959) A low Na K ATPase activity has also been observed in brain tissue from newborn rats (Samson and Quinn 1967)

The observed decreased effect of angiotensin on the ATPase of old animals may be related to a lower kidney function usually observed in aging animals

This work was supported by grants to Professor Bertil Josephson from the Swedish Medical Research Council (Grant B67 19X 573 02) Magnus Bergvalls Stiftelse and from Svenska liv forsakringsbolags namnd for medicinsk forskning and by a grant to Dr Klas Levin from the City of Stockholm

References

BARNETT H L and J VESTERDAL The physiological and clinical significance of immaturity of kidney function in young infants *J Pediat* 1953 *42* 99 119

BOŽOVIĆ L and J CASTENFORS Effect of ganglion blocking on plasma renin activity in exercising and pain stressed rats *Acta physiol scand* 1967 *70* 290 292

BUNAG R D I H PAGE and J W MC CUBBIN Neural stimulation of release of renin *Circulat Res* 1966 *19* 851 858

CASTENFORS J Effect of ethacrynic acid on plasma renin activity during supine exercise in normal subjects *Acta physiol scand* 1967 *70* 215 220

CHIGNELL C F and E TITUS Effect of adrenal steroids on a Na and K requiring adenosin [illegible] from [illegible] kidney *J biol Chem* 1966 *241* 5083—5089

K) activated ATP hydrolyzing enzyme system III and the supply of sodium on the enzyme system. 212 224

LANDON E J, N JAZAB and L FORTE, Aldosterone and sodium potassium dependent ATPase activity of rat kidney membranes *Amer J Physiol* 1966 *211* 1050—1056

LEVIN K The stimulating effect of angiotensin and vasopressin on adenosintriphosphatase activity in vitro *Acta physiol scand* 1970 *79* 37—49

LOWRY O H N R ROBERTS M L WU, W S HIXON and E J CRAWFORD The quantita [illegible] *Neurochem* 1967 *14* 421—427

SOKABE H, A MIKASA H YASUDA and G M C MASSON Adrenal cortex and renal pressor function *Circulat Res* 1963 *12* 94—100

WINBERG J, Determination of renal concentration capacity in infants and children without renal disease *Acta paediat* (Uppsala) 1959 *48* 318—328

Acta physiol. scand. 1970. 79. 58—63

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Pregnancy in Exercised and Food Restricted Goldthioglucose Obese Mice

By

N EINER-JENSEN, S LARSSON, P ALSTRUP NIELSEN AND N J SECHER

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Abstract

EINER-JENSEN N S LARSSON, P ALSTRUP NIELSEN and N J SECHER *Pregnancy in exercised and food restricted goldthioglucose obese mice* Acta physiol scand 1970 79 58—63

NMRI/BOM mice made obese with goldthioglucose were studied with respect to the influence of forced muscular exercise and food restriction upon fertility The obese mice were found to be sterile Only minor disturbances were observed in the vaginal smears After exercise and food restriction resulting in pronounced decrease in weight they became fertile and gave birth to normal litters

Mice made obese by a single injection of goldthioglucose (GTG) are unable to produce litters *(cf* Mayer 1963) According to Liebelt *et al* (1966) female mice treated with goldthioglucose show persistent vaginal cornification The same workers suggest that this finding is correlated to the extent of hypothalamic damage It is also known that electrolytical damage in the ventromedial parts of the hypothalamus will produce not only obese but also sterile animals According to Kennedy (1963) this is due to a damage also of structures concerned with the regulation of motivation and the anterior part of the pituitary gland On the other hand Mayer (1955) has suggested that goldthioglucose produces a selective chemical damage of glucoreceptors located in the satiety part of the 'feeding centre'

The present study was made to investigate the influence of reduced food intake and increased muscular activity upon the sexual cycle and fertility in female mice made obese by the injection of goldthioglucose

Material and methods

14 female NMRI/BOM mice weighing on the average 30 g starved over night were injected i p with 1 5 mg goldthioglucose (Schering AG) per g b w GTG was suspended in sesame oil 6 non injected animals were used as controls The animals were kept individually in single cages after the injection of GTG They had free allowance to tap water and a commercial mouse and rat pellet diet (Karensmolle® Denmark) and the food intake of the animals were registrated twice a week Immediately after injection of GTG great care was taken to facilitate the food and water intake for the mice The temperature of the animal room was 23° C and the humidity about 50 % The artificial light of the room was automatically regulated with light from 6 a m to 6 p m Vaginal smears were taken every morning nine days before the in

jection of GTG and daily afterwards The vaginal smears were taken with glass pipette in a drop of saline and examined directly without staining The mice were regarded as obese according to the parameters given by Liebelt *et al* (1966) and Larsson (1967) In reality this means a body weight over 40 g Therefore, the mice were divided into three groups, obese, injected non-obese and controls

After 2 months when the body weight had reached a plateau the obese, injected non-obese and control mice were subjected to forced exercise (Larsson 1967) After a training period the

lactation and the growth rate of the litters were recorded

Results

Of the 14 injected mice 3 animals died during the first and second days after injection

Body weight

From one week after the injection the GTG mice showed a marked increase in weight, reaching an average body weight of 46 g after one month and a plateau of approximately 51 g as an average after two months When dividing the animals into the groups mentioned with a final body weight of above and below 40 g the average weight was 60 g for 7 obese GTG mice and 38 g for 4 non-obese GTG mice 2 months after injection Six controls weighed in average 29 g (Fig 1)

Having started the forced exercise and the restricted feeding the mean body weight of the obese GTG mice decreased to 46 g during 1 month The mean body weight of the non-obese GTG animals decreased to 29 g and the weight of the controls was unchanged

Fig 1 Mean body weight for 7 obese GTG injected mice 4 non obese GTG mice and ■ control mice The vertical lines given indicate range

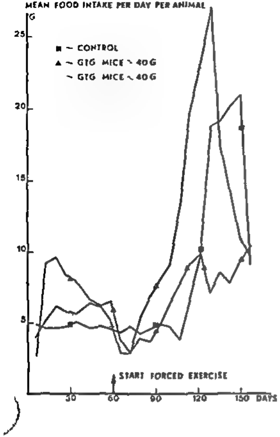

Fig 2 Mean food intake per day per animal for 7 obese GTG injected animals 4 non obese GTG mice and 6 control animals

After discontinuation of food restriction and forced exercise during and after pregnancy and lactation the body weight raised somewhat higher than before exercise

Food Intake

During the first 7 days after the injection the food intake of the GTG mice was smaller than the consumption of the controls Two or three weeks after injection the GTG mice ate on the average twice as much as the controls Later the food intake decreased somewhat (Fig 2)

All 3 groups of animals showed a marked decrease of food intake during the first days after forced muscular activity The largest decrease was shown by the obese GTG mice where the intake decreased from 6 4 g to 1 7 g per animal per day One week after forced muscular activity the intake of the controls was back to preexercise values while both GTG groups did not eat all the offered food (4 g) until two weeks after start Towards the end of pregnancy and during lactation the food intake was high in all 3 groups After lactation when the GTG mice still had free allowance to food, the intake arrived at a level corresponding to their intake before exercise and food restriction

Sexual Cycle

Only one animal showed tendency to persistent vaginal cornification and only for 10 days In the major part of the animals the vaginal smears showed a nearly regular sexual cycle in both the initial period after injection and in the period when they were subjected to forced activity (Table I)

Breeding

10 out of 11 GTG mice became pregnant after caging males and females together The conceptions occurred from the 1st to the 69th night after the first caging The mean period between caging and mating was 42, 18 and 2 days respectively for obese GTG mice, non-obese GTG mice and control animals Table I) The mean body weight on the day of conception was 43 29 and 30 for the three groups

One obese GTG mouse did not become pregnant and another ate all youngs The other 5 obese GTG mice gave birth to 35 (mean 7 0) youngs 21 of these were alive when being weaned The figures for the non obese GTG injected mice were 37 (mean 9 3) and 26 respectively and for the controls 45 (mean 7 5) and 41 There was no difference between mean growth rate of the youngs in the three groups (Table I)

Discussion

The present experiments show that in obese NMRI/BOM mice the injection of GTG in dosages inducing obesity does not need to cause persistent hypothalamic damage in structures regulating the sexual activity as suggested by Liebelt *et al* (1966)

The pregnancy rate in the GTG mice was the same as the control mice while pregnancy of the obese animals were delayed compared with the controls The effect of muscular exercise followed by reduction of body weight points towards two possibilities viz the obese female mice should anatomically be restricted to receive male mice and the disturbance of the endocrinological regulation followed by or resulting in increased fat content of the body *per se* could have caused the infertility

Liebelt *et al* (1966) described the development of persistent estrus in vaginal smears after GTG treatment but in our experiment the frequency of cornifications in GTG mice is close to or lower than that of the controls For these and for the discrepancy between our high pregnancy rate and the described infertility in obese mice the explanation may be the strain differences which have been reported after injection of GTG (Larsson 1967) or limitation of food intake before injection causing a more selective influence on hypothalamus with less damage to the centres for the releasing factors to the pituitary gland Kennedy and Mitra (1963) showed that electrolytic lesion in the lateral part of the ventromedial nucleus of the hypothalamus in the rat sometimes disturbed the estrus cycle but not more frequently than lesions which failed to cause obesity They found however, that ventromedial lesions will prevent mating behaviour even when they did not disturb the estrus cycle and they concluded that the ventromedial nucleus appears to play an essential part in mating

TABLE I Vaginal cycle, distance between caging and mating, breeding results and growth rate of

	Number of heats per week		Days between caging and mating
	before exercise	after exe till pregnancy	
Obese GTG	1 2	1 0	17
	0 7	0 6	73
	1 0	0 4	43
	1 2	0 9	70
	1 7	—	6
	2 6	1 5 (not pregn)	—
	0 9	1 8	43
Mean	1 3	0 9	42 0
Non obese GTG	1 1	—	0
	1 3	0 5	20
	1 2	0 5	29
	1 3	2 0	21
Mean	1 3	1 0	17 5
Control mice	0 7	0 6	3
	2 6	1 6	1
	1 3	1 4	4
	1 7	2 2	2
	2 3	2 2	2
	2 3	0 3	*
Mean	1 8	1 4	2 4

* First male was infertile ** Killed by the mother

behaviour but not in the regulation of gonadotropin release Averill and Purves (1963) found in pubertal rats with bilateral thermal lesions in the hypothalamus neither induced ovarian atrophy nor impared reproduction but failure of lactogenesis after otherwise normal pregnancies They found that the most likely explanation was that bilateral destruction of hypothalamic structures in the lateral preoptic area and in the lateral areas immediately posterior to the optic tracts has interfered with production or with release of prolactin from the anterior lobe of the pituitary The lactogenesis in our experiment as measured by increase of weight in offsprings seems undisturbed

If the hypothalamic damage in the GTG obese mice had also involved the structures regulating mating and the anterior pituitary it is unlikely that this damage should disappear due to forced muscular exercise and restriction in food intake As to the question of 'anatomical inability it seems as obese GTG females would not

the youngs in obese and non-obese goldthioglucose mice and control animals

Body weight (g) when		Number of youngs		Mean weight (g) of youngs days after birth		
starting exercise	mating	born	when being weaned	7	14	21
43 6	33 3	3	2	3 8	8 8	12 6
65 5	42 5	10	8	5 3	9 2	14 2
67 9	47 5	8	0	—	—	—
67 5	41 6	**	0	—	—	—
56 2	50 0	6	6	ca 5 0	10 2	15 4
62 9	—	—	—	—	—	—
56 3	44 0	8	5	5 7	11 4	17 4
60 0	43 3	7 0	4 2	5 0	9 9	14 9
35 0	35 0	10	0	—	—	—
35 7	26 5	11	10	4 4	8 4	13 (
41 0	28 6	8	8	5 0	9 2	13 9
34 7	24 6	8	8	5 4	9 6	14 7
36 6	28 7	9 3	6 5	4 9	9 1	13 9
26 9	27 4	5	4	5 8	11 0	16 5
25 7	25 9	8	7	5 6	9 8	16 4
34 1	30 1	8	8	5 7	10 0	15 5
26 3	28 7	10	9	4 0	7 6	10 7
28 9	30 5	5	5	5 5	10 6	15 8
32 4	35 5	9	8	3 6	—	—
29 1	29 7	7 5	6 8	5 0	9 8	14 8

cause a hinder for fertile non-obese males The question then arises as in the question of the etiology of the relative inability to produce litters in GTG obese females

References

Averill R. L. and H D Purves Differential Effects of Permanent Hypothalamic Lesions on Reproduction and Lactation in Rats *J Endocr* 1963 *26* 463—4 7

Kennedy G G The Hypothalamic Control of food intake *II Symp Swed Nutr Found* 1963 56—66 Ed G Blix Almqvist & Wiksell Stockholm

Kennedy B C and J Mitra Hypothalamic Control of Energy Balance and the Reproductive Cycle in the Rat *J Physiol* (Lond) 1963 *166* 395—407

Larsson S Factors of Importance for the Etiology of Obesity in Mice *Acta physiol scand* 1967 Suppl 294 1—87

L … G Liebelt Persistent Vaginal Cornification in *Endocrinology* 1966 *78* 845—854

… and the Body Weight the Glucostatic Theory *ad Sci* 1955 *63* 15—42

II Symp Swed Nutr Found 1963 29—

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	2 6	1 6	1
	1 3	1 4	4
	1 7	2 2	2
	2 3	2 2	2
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35 0	35 0	10	0	—	—	—
35 7	26 5	11	10	4 4	8 4	13 1
41 0	28 6	8	8	5 0	9 2	13 9
34 7	24 6	8	8	5 4	9 6	14 7
36 6	28 7	9 3	6 5	4 9	9 1	13 9
26 9	27 4	5	4	5 8	11 0	16 5
25 7	25 9	8	7	5 6	9 8	16 4
34 1	30 1	8	8	5 7	10 0	15 5
26 3	28 7	10	9	4 0	7 6	10 7
28 9	30 5	5	5	5 5	10 6	15 8
32 4	35 5	9	8	3 6	—	—
29 1	29 7	7 5	6 8	5 0	9 8	14 8

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References

AVERILL R L and H D PURVES Differential Effects of Permanent Hypothalamic Lesions on Reproduction and Lactation in Rats *J Endocr* 1963 *26* 463—477

KENNEDY G G The Hypothalamic Control of food intake *II Symp Swed Nutr Found* 1963 56—66 Ed G Blix Almqvist & Wiksell Stockholm

KENNEDY B C and J MITRA Hypothalamic Control of Energy Balance and the Reproductive Cycle in the Rat *J Physiol* (Lond) 1963 *166* 395—407

LARSSON S Factors of Importance for the Etiology of Obesity in Mice *Acta physiol scand* 1967 Suppl 294 1—82

LIEBELT R A K SEKIJA S ICHINOE and A G LIEBELT Persistent Vaginal Cornification in ... *Endocrinology* 1966 *78* 845—854

... d the Body Weight the Glucostatic Theory ... *d Sci* 1955 *63* 15—42

... *II Symp Swed Nutr Found* 1963 ...

Acta physiol. scand 1970 79 64—75

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Osmotic Factors Determining the Release of Catecholamines from Isolated Chromaffin Cell Granules

By

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Abstract

LISHAJKO, F *Osmotic factors determining the release of catecholamines from isolated chromaffin cell granules* Acta physiol scand 1970 79 64—75

On incubation of bovine adrenal medullary granules in isotonic sucrose KCl NaCl or K phosphate noradrenaline (NA) is initially released at a rapid rate followed by a slow exponential release Adrenaline (A) release in the same media does not show the rapid initial phase In hypotonic media both NA and A show a rapid initial release while in hypertonic solutions both NA and A are released at a constant slow rate ($t/2 = 115$—185 min at 7°) Partial replacement of sucrose or dextrose media by NaCl KCl or K phosphate decreases the release rate of NA and A Addition of 6 mM Mg^{2+} or Ca^{2+} prevents the initial rapid fall of NA and of A in hypotonic solution ATP Mg^{2+} inhibits the release rate of catecholamines in chloride free media more than ATP or Mg^{2+} alone The granules were shown to shrink in the presence of divalent ions as well as on addition of ATP or ATP Mg^{2+} During partial lysis ...
suggest that the NA ...
pressure than that ...
ATP complex The ...

Hillarp and Nilson (1954) have shown that isolated granules from adrenal medullary cells slowly release catecholamines (CA) in isotonic sucrose or in KCl or NaCl but rapidly lose the amines in hypotonic media The amine release occurred not only when the granules were transferred from isotonic to hypotonic media but also on changing hypertonic media to a less hypertonic or isotonic solution at +3° C No essential differences between noradrenaline (NA) and adrenaline (A) containing granules were observed Moreover Hillarp (1958 a) found that the chromaffin granules have an ATPase activity which is stimulated by Mg^{2+} ions confirmed by Banks (1965) Carlsson Hillarp and Waldeck (1963) showed that ATP Mg^{2+} stimulated CA uptake in chromaffin cell granules and that this uptake was influenced by a number of agents some of which also inhibited or enhanced the release Oka *et al* (1966) observed an initial rapid fall of NA during incubation of chromaffin cell granules in a sucrose TRIS medium This finding has been con

firmed (Lishajko 1969 b) and in addition it was shown that the granules with the initial rapid NA release rate were localized in the heavy granules fraction

In the present report the effects of ambient osmotic pressure, mono and divalent metal ions, and of ATP in non lysed and partially lysed granules have been studied on the amine release Also changes in optical density indicating swelling and shrinkage in the presence of Mg^{2+} and Ca^{2+} ions and ATP Mg^{2+} in isolated bovine adrenal medullary granules have been studied and the effect of osmotic factors on amine release discussed

Methods

Bovine adrenal glands were obtained from the slaughter house and transported at ice temperature to the laboratory within 30—60 min after the death of the animal All the subsequent preparations were made at 2—5° C The adrenal medulla was homogenized according to Potter—Elvehjem in 270 mM sucrose 160 mM KCl 130 mM K phosphate buffer or sucrose +20—40 mM TRIS-maleic acid buffer at pH 7.5 (100 mg/10 ml medium) The suspension was centrifuged at 1000×g for 10 min, and the granule-containing supernatant used for the experiment In other experiments the granule suspension after centrifugation at 1000×g was recentrifuged at 50 000×g for 30 min and the tube containing the high speed sediment washed with ice-cold 270 mM sucrose K phosphate or sucrose + TRIS buffer and the sediment resuspended in fresh medium The average amine content of 8 ml of the granule suspension from bovine adrenals was 142±6 µg NA and 351±18 µg A n=19) Incubation was performed without shaking After centrifugation the walls of the tube were washed and wiped dry and the sediment extracted with 0.5 ml 0.4 N perchloric acid The precipitate was spun down by centrifugation 5—10 min at 15 000×g NA and A were estimated fluorimetrically without adsorption on Al_2O_3) (Euler and Lishajko 1961 The optical density was measured at 550 mµ in a Beckman DU spectrophotometer using the granule free medium as reference ATP was determined by a modification of the luciferase method of Strehler and Totter (1954)

Results

1 *Isotonic media*

On incubation in isotonic media (270 mM sucrose 130 mM K phosphate or 160 mM KCl) at pH 6.7 the granules show a rapid initial release of NA 37, 25 and 25 per cent respectively in 5 min at 37° C This was followed by a slow exponential release in all three media with a half time (t/2) of 105 175 and 83 min respectively In contrast to NA A is released at a constant rate during the whole incubation period with a t/2 of 110 160 and 83 min respectively (*cf* Fig 1 and Table I) The release rate for NA and A between 5 and 60 min incubation period in the different media was thus about the same

2 *Hypotonic media*

In hypotonic media (210 mM sucrose 100 mM K phosphate and 130 mM KCl) the granules lose A as well as NA at a rapid initial rate (Table I and Fig 1) followed by a slow constant release rate with a t/2 for NA of 95 205 and 120 min and for A 105 105 and 305 min in the different media

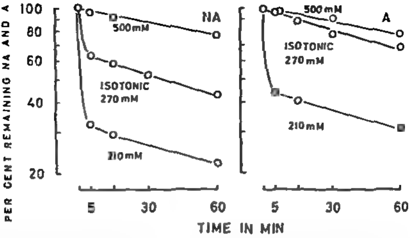

Fig 1 Bovine adrenal medullary granules resuspended and incubated in hypotonic (210 mM), isotonic (270 mM) and in hypertonic (500 mM) sucrose at pH 6.7 and 37° C Ordinate Per cent remaining noradrenaline (NA) and adrenaline (A) in sediment after incubation *Abscissa Incubation time in min*

3 *Hypertonic media*

When granules were incubated in hypertonic media (500 mM sucrose, 160—220 mM K-phosphate or in 240—280 mM KCl) at pH 6.7 at 37° C, both NA and A were released at a slow rate during the whole incubation period Thus only 2—5 per cent of amines were released in 5 min at 37° C, with a t/2 for both NA and A of

TABLE I Bovine adrenal medullary granules incubated in different concentrations of sucrose K phosphate and KCl pH 6.7 at 37° C Release of NA and A in per cent of original amount

Media	Conc in mM	Incub 5 min NA	A	Incub 5—60 min NA	A
Sucrose	210	68	56	10	13
	270	37	4	19	28
	400	22	3	17	21
	500	4	3	19	19
K phosphate	100	64	35	6	21
	130	25	7	15	20
	160	6	5	17	18
	220	4	2	16	18
KCl	130	74	66	7	4
	160	25	9	28	34
	200	16	6	25	26
	240	5	3	28	26
	280	[illegible]	3	23	24

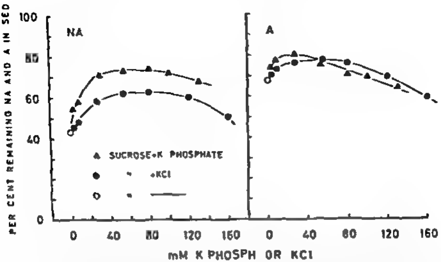

Fig 2 Resuspended bovine adrenal medullary granules incubated in 270 mM sucrose and in sucrose replaced with equiosmolar K phosphate (130 mM) or KCl (160 mM) at pH 6 7 for 60 min 37° C In sucrose alone (o) sucrose + K phosphate (▲—▲) and in sucrose + KCl (●—●) Ordinate Per cent remaining NA and A in sediment after incubation Abscissa Sucrose replaced with equiosmolar amounts of K phosphate or KCl in mM

165 185 and 115 min in the different media In 400 mM sucrose there is still an increased initial release of NA however (Table I) The absolute release rates of NA and A were 0 33 per cent/min both for NA and A in K phosphate buffer

An interesting difference was noted between isotonic sucrose and dextrose in that the release rate for both NA and A was considerably greater in the dextrose medium (Fig 3)

4 *Sucrose partially replaced by equiosmolar K phosphate or KCl*

When 270 mM sucrose was partially replaced by equiosmolar concentrations of K phosphate or KCl at pH 6 6—6 7 the release rate decreased in comparison with sucrose alone more for NA than for A (Fig 2)

From the kinetic data on NA and A release rate in Fig 3 it is seen that the presence of electrolytes retards the release in dextrose for A and in dextrose or sucrose for NA

5 *Medullary cell cytoplasm as incubation medium*

A preparation largely consisting of cell cytoplasm was obtained from bovine adrenal medullary cells by squeezing the organ between rollers After centrifugation at $1000 \times g$ for 1—2 min incubation was performed in 50 µl pipettes at 37 and pH 6 4—6 6 After incubation 130 mM K phosphate pH 7 5 was added to the suspension followed by centrifugation for 30 min at $50\,000 \times g$ The tube was washed with fresh K phosphate and the sediment used for analysis of NA and A In the undiluted

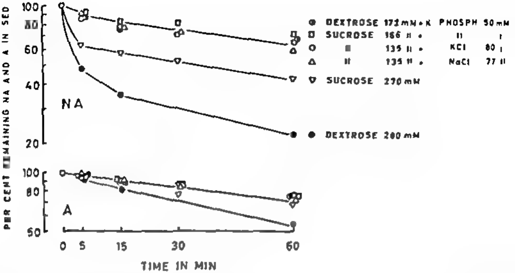

Fig 3 Resuspended bovine adrenal medullary granules incubated in 270 mM sucrose or 280 mM dextrose and after partial replacement with equiosmolar amounts of K phosphat, KCl or NaCl pH 6 6—6 8 at 37° C Ordinate Per cent remaining NA and A in sediment Abscissa Incubation time in min Each point is the mean of 1—3 expts

medium NA and A are released at the same slow rate ($t/2=230$ min), similar to that in 190 mM K phosphate Dilution of the medium with water 1 1 1 only slightly increased the amine release while dilution to 1 1 25 caused a rapid loss of 50 per cent of NA as well as A into the media at 37° C On continued incubation no further release of NA and A was observed for 60 min at 37° C

6 *Influence of Mg^{2+} and Ca^{2+} ions*

Addition of 6 mM $MgCl_2$ moderately lowers the release rate for NA in isotonic sucrose and phosphate but has less action on A release 6 mM Ca^{2+} inhibits NA and A release in sucrose but not in KCl and NaCl In K phosphate 6 mM Ca^{2+} produces a precipitate leaving a smaller quantity of CA in the sediment (*cf* Table II) However, addition of 6 mM $MgCl_2$ or $CaCl_2$ in hypotonic sucrose (0 78×isotonic) strongly inhibits the initial rapid release of NA and A as does 54 mM choline chloride (Fig 4) No action of Ca^{2+} or Mg^{2+} is observed in hypertonic media

7 *The effects of Mg^{2+} and Ca^{2+} on optical density of the granule suspension*

The effects of Mg^{2+} and Ca^{2+} on the CA release suggest a membrane effect On testing the optical density (O D) at 550 mμ an increase of about 85 per cent for both Ca^{2+} and Mg^{2+} was observed while in the control the O D decreased by 5 per cent in 5 min at 37° C and 16 per cent after 60 min (Fig 5) The initial rapid fall in O D is synchronous with the rapid fall of the NA content during incubation suggesting an equilibration of osmotic pressure of the granules

Shrinkage appears to occur from a concentration of 0 3 mM Mg^{2+} ion and reaches

TABLE II Isolated bovine adrenal medullary granules resuspended and incubated for 5 and 60 min at 37° C in 270 mM sucrose 130 mM K phosphate, 160 mM KCl and in 155 mM NaCl pH 6 7—6 8 alone and with addition of Mg^{2+} and Ca^{2+} Remaining NA and A in sediment in per cent of original amount M±SEM

Media	Incub time in min	No addition		+6 mM Mg^{2+}		+6 mM Ca^{2+}	
		NA	A	NA	A	NA	A
Sucrose	5	61±2	93±3	87±3	96 4	85	90
	60	51±6	65±2	80±4	77 2	77±3	70±2
K phosphate	5	75±2	96±2	82±5	99±1	27±2	40±7
	[illegible]	57±2	67±2	73±6	77±2	9±5	15±6
KCl	5	71±3	89±2	76±4	96 4	67	85
	60	46±2	55±2	51±1	6 1	46	54
NaCl	5	73	92	83	100	74	86
	60	46	58	51	77	48	53

a maximum (plateau) at a concentration of 6 mM Mg in the incubation medium Choline chloride 54 mM also induces shrinkage of the granules and increases the O D from 0 70 to 0 79 in 5—60 min at 37° C while addition of 54 mM KCl to the suspension fluid had a smaller effect In contrast prenylamine which releases catecholamines at 3×10^{-5} M (Schone and Lindner 1962 Carlsson *et al* 1963 Euler *et al* 1964) accelerates the swelling of the granules and decreases O D from 0 70 to 0 62 in 5 min at 37° C A fall in O D as a result of partial hyposmotic lysis is likewise accompanied by a liberation of catecholamines (Hillarp and Nilson 1954)

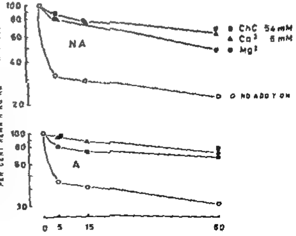

Fig 4 Resuspended bovine adrenal medullary granules incubated in 210 mM sucrose at pH 6 6 37° C alone and with addition of 54 mM choline chloride (ChCl) 6 mM $MgCl_2$ or $CaCl_2$ No addition (O—O) addition of ChCl (■—■) $CaCl_2$ (▲—▲) and $MgCl_2$ (●—●) Ordinate Per cent remaining NA and A in sediment Abscissa time in min

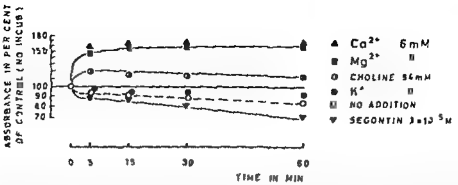

Fig 5 Bovine adrenal medullary granules incubated in 270 mM sucrose at pH 6 6—6 7 37° C No addition 0—0), addition of 6 mM $CaCl_2$ (▲—▲), 6 mM $MgCl_2$ (■—■), 54 mM choline chloride (◑—◑), 54 mM KCl (●—●) and 3×10^{-5} M Segontin® (▼—▼) Ordinate Absorbance in per cent of non incubated (+2° C) at 550 mμ Abscissa Incubation time in min

TABLE III Bovine adrenal medullary granules non lysed and after partial lysis 2—5 min in 210, 170 and 135 mM sucrose at +2° C incubated in 270 mM at 37° C alone and with 6 mM $MgCl_2$ Optical density (O D) at 550 mμ, NA, A, ATP on per cent of original values and molar ratio (CA/ATP) in non lysed and lysed granules (left table), and optical density after incubation for 5 and 30 min at 37° C alone and in the presence of Mg^{2+} (right table)

Sucrose	Before incubation					After incubation in 270 mM sucrose				
in mM						Incub time in min	No addition		+ Mg^{2+}	
	O D	NA	A	ATP	Molar ratio CA/ATP		O D	%	O D	%
	Non lysed									
270	100	100	100	100	4 5	—	0 60	100		
270						5	—	—	0 885	147
270						30	0 543	90	0 965	161
	Partial lysis before incubation									
210	92	80	80	91	4 0	5	—	—	0 815	136
210						30	0 43	72	0 89[illegible]	149
170	62	26	11	17	4 5	5	—	—	0 613	102
170						30	0 345	[illegible]	0 675	113
135	54	10	2	6	4 0	5	—	—	0 563	94
135						30	0 30	50	0 625	104

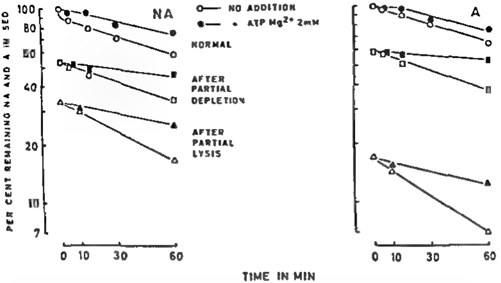

Fig 6 Bovine adrenal medullary granules resuspended and incubated in 270 mM sucrose +40 mM TRIS maleic acid buffer at pH 7 5 37° C, without addition (empty circles) and with addition of 2 mM ATP Mg^{2} (filled circles), untreated after partial depletion and after partial lysis Ordinate Per cent remaining NA and A in sediment Ascissa Incubation time in min

Table IV Bovine adrenal medullary granules incubated in 270 mM sucrose + 40 mM TRIS maleic acid buffer pH 7 5 at 37° C

a) untreated

b) after partial depletion (incubated for 60 min at 37 C sediment resuspended in fresh media)

c) after partial lysis (0 2 ml water added to 0 5 ml granule suspension and sucrose + TRIS subsequently added) alone and in the presence of 2 mM ATP Mg^{++}

Remaining NA and A in sediment in per cent of original amount

	Incub time in min	No addition NA	No addition A	+ATP Mg^{++} NA	+ATP Mg^{++} A
Untreated	5	87	98	95	97
	15	79	90	95	99
	30	70	[illegible]	81	[illegible]
	60	58	65	75	76
After partial depletion	60	[illegible]	59	—	—
	60+5	50	57	50	58
	60+15	45	51	48	57
	60+60	35	37	47	53
After partial lysis	—	33	18	—	—
	10	29	14	30	15
	60	16	7	25	

TABLE V. Bovine adrenal medullary granules incubated in 270 mM sucrose + 20 mM TRIS maleic acid buffer pH 7.5 60 min at 37° C alone, with ATP and both ATP+Mg^{++}, Ca^{++} and K^{+}. Remaining NA and A in sediment in per cent of original amount. Optical density at 550 mμ after incubation

		Added ions in mM	Number of expts	Per cent NA	A	O.D.	O.D. in %
Control +2	non incub	—		100	100	0.80	100
»	incub	—	12	55±2	62±1	0.63	79
ATP 3 mM		—	8	63±3	80±2	0.74	93
» »	Mg^{++}	3	3	80±9	86±5	0.84	105
» »	$+Ca^{++}$	3	3	79±5	74±8	0.81	101
» »	K	54	3	74±8	76±4	0.77	96

The effect of Mg on swelling and shrinkage in partially lysed granules is shown in Table III. The granules were suspended in 270 mM sucrose and lysed by addition of water to reduce the sucrose concentration to 210, 170 and 135 mM respectively at +2° C. The catecholamine content fell during lysis for 2—5 min and subsequent reconstitution of isotony with sucrose to 80, 17 and 5 per cent and ATP *to 91, 17 and 6 per cent respectively of the original amount. The molar ratio* CA/ATP was 4.5 in non lysed and 4.0, 4.5 and 4.0 in lysed granules. The results confirm previous findings that during lysis catecholamines and ATP are released in approximately stoichiometric proportions.

Addition of 6 mM Mg^{2+} to non lysed as well as previously lysed granules caused the granules to contract and the O.D. at 550 mμ to increase except after the more *effective lysis in 135 mM sucrose (see Table III).*

II. Effect of ATP-Mg^{2+} on the amine release in normal, partially depleted and lysed granules

In view of the presence of ATP in granules it appeared of interest to study if addition of ATP-Mg^{2+} influences the release rate of catecholamines. As seen in Fig. 6 addition of 2 mM ATP-Mg^{2+} inhibits the release of NA as well as of A in sucrose-TRIS buffer (*cf.* Table IV). An inhibitory effect of ATP-Mg^{2+} was also seen in *hypotonic, isotonic and hypertonic K phosphate and after partial depletion and* lysis in hypertonic sucrose.

The inhibition of release caused by ATP is accompanied by an increase in O.D. at 550 mμ by 15 per cent during incubation for 60 min at 37° C (Table V). $MgCl_2$ or $CaCl_2$ further increases O.D. while 54 mM KCl had a smaller effect.

Discussion

The striking difference in the initial rate of release of NA and A from adrenal medullary granules in isotonic media (OKA *et al.* 1966, Lishajko 1969 b) raises the question of the reason for this behavior.

The present findings that A is also released at a rapid rate initially if exposed to hypotonic media, while NA assumes a slow and constant release rate in hypertonic media suggest that the NA granules in the chromaffin cells exist in an osmotic concentration higher than that of the isotonic media used during incubation. When granules are transferred to a medium of lower osmolarity than in the granules themselves a lytic effect might occur which could explain the initial fall of NA (*cf* Hillarp and Nilson 1954). The granules then swell until osmotic equilibration has been reached as indicated by the fall in optical density. The previous finding that NA granules are localized in the heavy fraction is in harmony with this assumption.

Since the A granules show no rapid release phase in isotonic media it might be assumed that they are in equilibrium with the medium from the start. On the other hand lysis by exposure to hypotonic media affects the release of A slightly more than that of NA (Fig 6 Table IV). In partially depleted granules no initial fall of NA occurs, suggesting that osmotic equilibration of the granules has already occurred during the previous incubation period (Fig 6 Table IV). The small degree of swelling during incubation at 37° C after partial lysis also suggests rapid equilibration (Table III).

A possible explanation of the apparent hypertonicity in the NA containing granules could be that the postulated Mg^{2+} NA ATP complex (Hillarp 1958 b Belleau 1960 Smith 1968) is less stable than the corresponding complex in the A granules. A dissociation of the complex would presumably lead to an increased osmotic pressure of the NA granules at the beginning of the incubation. An initial rapid osmotic equilibration of the NA granules with the medium would cause swelling and a fall in optical density (Fig 5) accompanied by a rapid fall of NA ATP and soluble protein. Previously (*cf* Lishajko 1969 b) it has been shown that a gradual increase in temperature of the granule suspension from +2° C up to 37° C in 20 min causes a rapid fall of Mg^{+} NA ATP and soluble protein.

After equilibration of the granules with the medium the same process might continue during further incubation with dissociation of the intra granular complex thereby increasing the osmotic pressure in comparison to the media followed by osmotic equilibration. It appears conceivable that such events might induce a continuous release of catecholamines ATP and soluble protein. The state of the granule membrane should also be of importance for the release rate. ATP hydrolysis may cause the granule membrane to contract causing inhibition of amine release as well as uptake of water. After out-diffusion of terminal phosphate and ADP into the media the membrane may relax and the release rate increase.

Since addition of ATP Mg^{-} to the granule suspension in isotonic K phosphate or sucrose +40 mM TRIS prevents swelling and the initial rapid fall of NA it might be assumed that a ATP Mg^{2+} dependent mechanism is operating in the chromaffin cell. This may maintain the granule membrane in a contracted state and protect granules from rapid osmotic shock *in vivo* and control the permeability of the granule membrane (Table V).

Electron microscopic data from chromaffin cells (Wassermann and Tramezzani

1963, Coupland 1966, Pohorecky and Rust 1968) seem to indicate that the NA granules are more electron dense than that A granules Oka *et al* (1967), Poisner and Trifaro (1967) and Trifaro and Poisner (1967) have reported that ATP Mg^{2} increases the release rate of catecholamines, protein and ATP and also cause a fall in O D It has subsequently been shown that chloride ions are essential for the release of catecholamines by ATP-Mg⁻ (Lishajko 1969 a) Chloride ions also induce the granules to swell in the presence of ATP-Mg^{2+}

Catecholamines and ATP are released after partial lysis in a molar ratio varying between 4—4 5 (see Table III) On the other hand Stjärne (1964) has shown that the fall of ATP was more rapid than that of CA in the presence of anion exchange resin in 0 5 isotonic K phosphate buffer

The absence of an initial fall of NA when granules are incubated in chromaffin cell cytoplasm and the subsequent slow release suggests that Mg^{2} and possibly other co-factors are present in the press juice in concentrations inhibiting the release of CA

From the results obtained it appears that two different types of effects exist as regards retardation of release of CA and other cell constituents Mg^{2+} and Ca^{+} ions in sucrose media seem to exhibit a stabilizing effect on the granules, probably by binding negative charges or by complex formation (chelating effect) in the granule membrane (Fig 5) (*cf* Weil-Malherbe and Posner 1963, Greenberg and Kolen 1966) Other divalent ions, Sr^{2}, Ba^{+}, Co^{+} and Mn^{+} act in a similar way in sucrose media On the other hand the effect of choline chloride, K or Na^{+} seems to be of another kind since these have to be added in about 10 times higher concentration as the divalent ions in order to cause the same inhibitory effects This effect like that of other monovalent ions as Li Cs⁺ H_4N may be of a more unspecific osmotic character

Conclusions

The difference in the initial release rate of NA and A on incubation of adrenal medullary granules in isotonic media suggests that NA and A granules have different osmotic pressure *in vitro*

Alterations in the optical density indicate that the granules shrink or contract in the presence of Mg^{2} and Ca^{+} ions in sucrose and also on addition of ATP The rapid initial release of catecholamines from granules under certain conditions appears to be associated with a splitting of intragranular components causing a rise in the osmotic pressure and swelling of the granules Reversely, inhibition or retardation of release may occur as a consequence of membrane tightening It is suggested that the same mechanisms operate in chromaffin cells even *in vivo*

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References

BANKS P The adenosine triphosphatase activity of adrenal chromaffin granules *Biochem J* 1965 *95* 490—496

... sites Ciba Foundation ...

... ATP dependent storage ... *hysiol scand* 1963 59 Suppl 215

COUPLAND R. E, The mechanism of the differential staining reaction for adrenaline and noradrenaline storing granules in tissues fixed in glutaraldehyde *J Anat* 1966 *100* 227—243

EULER, U ... and F LISHAJKO Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—355

EULER, U S v L. STJÄRNE and F LISHAJKO Effects of reserpine Segontin and phenoxybenzamine on the catecholamines and ATP of isolated nerve and adreno-medullary storage granules *Life Sci* 1964 *3* 35—40

GREENBERG R and A KOLEN Effects of acetylcholine and calcium ions on the spontaneous release of epinephrine from catecholamine granules Proceedings of the Society for *Exp Biology and Medicine* 1966 *121* 1179—1184

HILLARP, N A, Enzymic systems involving adenosinephosphates in the adrenaline and noradrenaline containing granules of the adrenal medulla *Acta physiol scand* 1958 a *42* 144—165

HILLARP, N A Adenosinephosphates and inorganic phosphate in the adrenaline and noradrenaline containing granules of the adrenal medulla *Acta physiol scand* 1958 b *42* 321—332

HILLARP N A and B NILSON The structure of the adrenaline and noradrenaline containing granules in the adrenal medullary cells with reference to the storage and release of the sympathomimetic amines *Acta physiol scand* 1954 *31* Suppl 113 79—107

LISHAJKO F Influence of chloride ions and ATP Mg⁺ on the release of catecholamines from isolated adrenal medullary granules *Acta physiol scand* 1969 a *75* 255—256

LISHAJKO F Release reuptake and net uptake of dopamine noradrenaline and adrenaline ... 159—171

... adrenaline and ... —438

... catecholamine ...

... Studies on the cortical control of the adrenal medulla in the rat *J Pharmacol exp Ther* 1968 *162* 227—238

POISNER A M and J M TRIFARO The role of ATP and ATPase in the release of catecholamines from the adrenal medulla I ATP-evoked release of catecholamines ATP and protein from isolated chromaffin granules *Mol Pharmacol* 1967 *3* 561—571

SCHONE H H and E LINDNER Über die Wirkung von N (3 Phenyl propyl (2)) 1 1-diphenyl propyl (3) amin auf den Katecholamine Stoffwechsel *Klin Wschr* 1962 *40* Jahrgang 1196—1200

SMITH A D ... granules *In* A Symposium on "The Internal ... mal Cells Ed ... Campbell 1968

ST ... and release mechanisms *Acta physiol* ...

ST ... of ATP and related compounds Firefly luminescence and other methods *In* Methods of Biochemical Analysis Ed D Glick 1954 *1* 341—356

TRIFARO J M and A M POISNER The role of ATP and ATP-ase in the release of catecholamines from the adrenal medulla II ATP-evoked fall in optical density of isolated chromaffin granules *Mol Pharmacol* 1967 *3* 572—580

WASSERMANN G and J H TRAMEZZANI Separate distribution of adrenaline and noradrenaline secreting cells in the adrenal of snakes *Gen Comp Endocr* 1963 3 480—489

WEIL MALHERBE H and H S POSNER The effect of drugs on the release of epinephrine from adrenomedullary particles *in vitro* *J Pharm exp Ther* 1963 *140* 93—101

Acta physiol. scand. 1970. 79. 76—87

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Effects of Monovalent Cations on Sodium Permeability of Human Red Cells

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Abstract

WIETH, J O *Effects of some monovalent cations on sodium permeability of human red cells* Acta physiol scand 1970 *79* 76—87

It has been shown previously that monovalent anions of the lyotropic series alter the cation permeability of human red cells In order to determine whether the anion induced effects on permeability are inspecific, the effects of an analogous series of monovalent cations were investigated The permeability to ^{22}Na was the same, whether the predominant cation of a bicarbonate medium was Li, Na, K, Rb, Cs or choline In chloride media total substitution of Na with Li did not change the permeability to ^{22}Na, whereas it was doubled after replacement with K Rb, Cs or choline Because both lithium and choline ions stabilize water structure whereas K Rb, and Cs are structure breakers, it is not likely that effect of ions is due to an inspecific breaking effect on water structure A number of distinct differences between the permeability changes caused by anions and by cations indicate that the mechanisms of interaction with the cell membrane must be different for negative and positive ions Additional information was obtained about the effect of bicarbonate on the cation selectivity of the red cell membrane Bicarbonate facilitates the permeation of hydrated monovalent cations as lithium and sodium whereas the permeability to potassium and cesium is reduced It is suggested that the increased influx is due to the formation of ion pairs ($LiCO_3^-$ and $NaCO_3^-$), penetrating the anion selective membrane much more readily than the cations

Sodium and potassium fluxes of human red cells have previously been shown to be influenced by various monovalent anions The monovalent anions of the Hofmeister series Cl, Br, NO$_3$, I and SCN increased the membrane permeability to both sodium and potassium whereas bicarbonate was found to increase sodium permeability selectively (Funder and Wieth 1967 b, Wieth 1969) The results suggested that the mode of action of bicarbonate was different from that of the other anions

Hippel and Wong (1964) and Robinson and Jencks (1965) have shown that lyotropic series of anions and cations may exert identical effects on the conformation of several macromolecules Their conclusion was that the structural changes caused by salts were a consequence of very general effects of various ions on th structure of

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solvent which in turn modify the solvent macromolecule interactions necessary for a stable configuration Such a non specific salt effect was found by Singer and Tasaki (1968), who showed that the lyotropic series of anions and cations produce identical and additive effects on the permeability of the squid axon Therefore, in the further exploration of the action of anions on cation permeability of the red cell membrane it is important to examine whether the effect of anions on red cell permeability is mimicked by an analogous series of cations In the present work red cell permeability to sodium was examined in the presence of the chlorides and bicarbonates of the following six ions lithium sodium potassium rubidium cesium and choline

The experimental results showed that the effects of monovalent anions on cation permeability are not due to properties which are shared by ions of the cationic series Further information was obtained about the bicarbonate induced selectivity towards cations by the finding that the permeability to both lithium and sodium is markedly increased when chloride is replaced with bicarbonate whereas bicarbonate does not increase the influx of potassium and cesium

Methods

Sampling of blood wash ng and incubation of red cells at 38° C and pH 7 40 were carried out accord ng to a technique previously described (Funder and Wieth 1967 a b) The isola

the two instruments (Beckman 4100 and Eppendorf) because repeated potassium and sodium analyses were carried out on the same pool of deep frozen red cell lysate With the Beckman photometer the following mean values and standard variat ons were obtained by 40 deter minations over a period of two years K 89 9 (S.D 2 ?) Na 13 6 (S D 0.37) meq per kg red cell lysate The corresponding figures for the Eppendorf photometer (from a one year period) were K 89 9 (S.D 1 7 n=11) Na 13 5 (S.D 0 60 n=2?) showing that identica results were obta ned with the two types of equipment Lithium in cells and med um was analysed with the Eppendorf photometer

Simultaneous determ nat on of the isotopes ^{22}Na and ^{36}Cl was carried out as described previously (Wieth 1969) The same reference conta ns informat on about the isotopes em ployed The

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in order to reach the same level of intracellular rad oactivity

The rate of sodium influx was employed as a measure of the sod um permeabil ty of the cells and was expressed by the relation

$$M_{n} = k_1 \cdot Na_o$$

where M_n denotes sod um influx (meq/kg cell solids hr k_1 is the rate constant and Na_o is the extracellular sodium concentration The experiments were carried out in the presence of ouabain (see below) The rate of sodium influx could be calculated from the in tial increase of intracellular ^{22}Na

$$k_1 = \frac{\text{cpm/kg cell sol ds}}{\text{cpm kg extracellular med um}} \cdot \frac{60}{t} \ (\text{hr}^{-1})$$

where t is the duration of the experiment (min

Cesium influx The isotope ^{137}Cs employed for the determination of cesium uptake in CsCl and $CsHCO_3$ media was obtained from AEK Riso Denmark as a carrier free solution of CsCl The amount of activity employed for experiments was 0 6 µC/ml cell suspension

Sulfate influx A comparison of the rates of $^{35}SO_4$ exchange in NaCl and KCl med a was performed to determine whether the substitut on of Na by K affects the anion permeab lity of

human red cells. Details about the experimental method are given by Wieth (1970 b). The media employed f[illegible] K_2SO_4 in addition to the ions listed in Table I. The [illegible] te medium at least 1 hr before the addition of tracer [illegible] l by means of the rate constant of sulfate exchange [illegible]. The rate constant is equal to the fraction of cellular sulfate exchanged per unit time.

Electrolyte media. At least 4 [illegible] donors were performed in each of the 12 media specified [illegible] ments were performed to study the interindividual variab[illegible] the relation between Na influx and extracellular Na/K and Na/[illegible] concentrations (Tables IV and V). The electrolyte media for these experiments were prepared by mixing adequate amounts of the media specified below.

The composition of the media is shown in Table I. X^+ represents one of the following six cations: Li, Na, K, Rb, Cs or choline. The media contained $3 \cdot 10^{-4}$ M ouabain. ^{22}Na and ^{36}Cl were added in a 0.15 M NaCl solution, whereby about 0.2 meq Na was added per litre of medium.

Sodium influx into human red cells increases appreciably at extracellular potassium concentrations below 5 mM (Glynn 1956, Garrahan and Glynn 1967). To avoid this effect the potassium concentration of the media employed in the present investigation was kept high (20 mM). [illegible]

TABLE I. The composition of the electrolyte media. X^+ represents one of the following cations: Li, Na, K, Rb, Cs or choline

	Chloride media mM	Bicarbonate media mM
X	142	142
Potassium	20	20
Calcium	1.5	1.5
Magnesium	1	1
Bicarbonate	22	162
Chloride	143	3
Phosphate	1.1	1.1
Glucose	5	5

Results

The effect of monovalent cations on sodium permeability

Table II demonstrates the effect of various monovalent cations on the rate of sodium influx into human red cells. The rate was not affected when NaCl or $NaHCO_3$ was replaced by LiCl or $LiHCO_3$. The mean values of the rate constants of sodium influx were doubled when NaCl in the medium was replaced by the chlorides of potassium, rubidium, cesium or choline, whereas the increase found when $NaHCO_3$ was replaced with the bicarbonates of the same four cations was only about 15 per cent. The differing rates of sodium influx found in the presence of sodium *bicarbonate* and sodium *chloride* have been described previously (Funder and Wieth 1967 b, Wieth 1969).

TABLE II *Effect of monovalent cations on the rate constant of sodium influx and on chloride distribution between cells and medium (pH 7.40, 38° C).* Sodium influx (meq/kg solids hr) = k_i Na_o, the extracellular sodium concentration, which was between 0.2 and 0.[illegible] meq/l except in the sodium media (*142 meq/l*). The media are described in the method section. The results are presented as the mean value and range of each experimental series.

Cation	Chloride media			Bicarbonate media		
	No. of expts	sodium influx (rate constant) k_i (hr^{-1})	^{36}Cl (cell water) / ^{36}Cl (medium)	No. of expts	sodium influx (rate constant) k_i (hr^{-1})	^{36}Cl (cell water) / ^{36}Cl (medium)
Lithium	5	0.053 (0.041—0.065)	0.64 (0.63—0.65)	9	0.127 (0.116—0.146)	0.54 (0.51—0.58)
Sodium	4	0.047 (0.045—0.052)	0.50 (0.48—0.52)	4	0.128 (0.117—0.141)	0.59 (0.57—0.61)
Potassium	7	0.116 (0.084—0.157)	0.54 (0.50—0.60)	4	0.155 (0.145—0.165)	0.58 (0.57—0.59)
Rubidium	4	0.109 (0.086—0.136)	0.59 (0.55—0.62)	4	0.150 (0.143—0.158)	0.54 (0.50—0.59)
Cesium	4	0.093 (0.072—0.120)	0.59 (0.55—0.62)	4	0.148 (0.142—0.153)	0.56 (0.53—0.59)
Choline	4	0.093 (0.078—0.105)	0.55 (0.48—0.60)	10	0.147 (0.130—0.164)	0.53 (0.52—0.55)

Extracellular sodium concentration was between 0.2—0.7 meq/l except in the NaCl and $NaHCO_3$ media (142 meq/l). The extracellular sodium concentration rose on an average 0.1 meq/l during the experiments. The intracellular sodium content of the cells was in all groups within the range of 17—20 meq/kg cell solids except in the sodium bicarbonate medium, where the mean value of 25.6 meq/kg cell solids at the start of the experiment was due to the increased rate of sodium influx in the equilibration period preceding the experiment.

The distribution of radioactive chloride between cell water and medium was determined, and the values are shown in Table II. Mean values of the ratio ^{36}Cl (cell water)/^{36}Cl (medium) were in all groups within the range 0.53—0.64. As the chloride ion distributes rapidly and passively across the red cell membrane the results show that the differing rates of sodium influx in the various media were not caused by diffusion potentials, a source of error which has been suggested to threaten permeability experiments of the type described here (Davson 1940).

The experiments shown in Table II were performed at 38° C. By analysing control samples kept at 0° C, it was established that none of the cations studied had the ability to facilitate sodium influx at low temperatures, an effect which is charac-

TABLE III The range of interindividual variation of the rate of sodium influx in chloride media (pH 7.40, 38° C). (Ch)Cl = choline chloride. The media are described in the method section. Sodium influx (meq/kg cell solids hr) = $k_i \cdot Na_o$ (Na_o = extracellular sodium (meq/l))

Medium	sodium influx (rate constant) k_i (hr^{-1}) Donor J. F.	per cent increase	sodium influx (rate constant) k_i (hr^{-1}) Donor J. W.	per cent increase
LiCl	0.065	} 0	0.045	} 0
NaCl	0.052		0.046	
KCl	0.157	168	0.084	85
RbCl	0.136	132	0.086	89
CsCl	0.120	105	0.080	76
(Ch)Cl	0.105	80	0.078	71

TABLE IV The rate of sodium influx at various levels of extracellular sodium and potassium. All the experiments were carried out with cells from Donor J. F. (pH 7.40, 38° C). The rate of sodium influx was not significantly increased until extracellular potassium concentration exceeded 55 meq/l

Medium	Date	Extracellular sodium (meq/l)	Extracellular potassium (meq/l)	Sodium influx (rate constant) k_i
Plasma	17.4.64	131	5.7	0.056
Plasma	27.5.64	137	4.7	0.056
Chloride	3.8.65	142	5.7	0.058
Ringer's solution	1.9.65	142	5.7	0.059
—	8.11.67	142	20	0.052
—	2.2.68	106.5	55	0.060
—	—	71	91	0.066
—		35.5	126.5	0.094
—	—	0.7	162	0.145
—	—	0.4	162	0.157

teristic for large monovalent anions like thiocyanate and salicylate (Wieth 1970 a).

The rate constants of sodium influx found in the KCl, RbCl, CsCl and choline chloride media (Table II) showed a much wider range of interindividual variation than was found in similar experiments where the anionic composition of the medium was varied (Funder and Wieth 1967 b). The greatest variations were found in the KCl medium. Therefore, repeated determinations were performed with two months interval on cells from two donors in order to evaluate the intraindividual variability. This was found to be small: the rate constant of sodium influx being 0.084 and 0.094 in the cells of one donor, 0.157 and 0.145 in the other donor's cells. The same two

TABLE V Sodium influx at various levels of extracellular lithium and sodium in chloride and bicarbonate media (pH 7.40 38° C) The media are described in the method section

Extracellular concentration (meq/l)		Rate constant of sodium influx k_1 (hr^{-1})			
Lithium	Sodium	Chloride medium		Bicarbonate medium	
		Donor J W	Donor J F	Donor J W	Donor A J
0	142	0.046	0.052	0.141	0.126
35.5	106.5	0.041	0.056	0.129	0.142
71	71	0.040	—	0.121	0.134
106.5	35.5	0.047	0.060	0.132	0.136
142	0.1—0.4	0.041	0.063	0.142	0.127

donors uniformly presented respectively the lowest and the highest rate constants of sodium influx in the chloride media The values are shown in Table III which demonstrates that not only the absolute magnitude of the rate constant but also the relative changes of sodium permeability were different in cells from the two individuals K^+ caused a maximum increase of sodium permeability in the cells of J F whereas K^+, Rb^+, Cs^+, and choline had almost identical effects on the cells of J W

The dependence of the rate of sodium influx on extracellular NaCl and KCl concentrations was examined on the cells which displayed the most pronounced sensitivity to the substitution of Na by K The results which for the sake of comparison also include experiments performed in plasma and in ordinary NaCl Ringer's solution (Funder and Wieth 1967 a, b), are shown in Table IV The rate of sodium influx was not affected by the substitution of plasma with an electrolyte medium of comparable composition Increasing extracellular potassium concentration from 5 to 20 meq/l caused an insignificant reduction of sodium influx at an extracellular sodium concentration of 142 meq/l The gradual substitution of sodium with potassium did not cause any marked increase of the rate of sodium influx until potassium concentration was above 100 and sodium concentration below 50 meq/l A similar dependence on concentration was observed when NaCl was gradually replaced by RbCl or CsCl

Lithium appeared to be the only cation which was able to replace sodium without affecting the rate constant of sodium influx (Table II) The results shown in Table V demonstrate that substitution of Na by Li was without effect on sodium permeability within the whole range of 0—142 meq/l both in chloride and in bicarbonate media

Sulfate permeability in NaCl and KCl media

The rate of sulfate exchange through the red cell membrane was determined i NaCl (4 expts) and KCl (4 ex

TABLE III The range of interindividual variation of the rate of sodium influx in chloride media (pH 7.40 38° C) (Ch)Cl = choline chloride The media are described in the method section Sodium influx (meq/kg cell solids hr) = k_1 Na_o (Na_o = extracellular sodium (meq/l))

Medium	sodium influx (rate constant) k_1 (hr^{-1}) Donor J F	per cent increase	sodium influx (rate constant) k_1 (hr^{-1}) Donor J W	per cent increase
LiCl	0.065	} 0	0.045	} 0
NaCl	0.052		0.046	
KCl	0.157	168	0.084	[illegible]
RbCl	0.136	132	0.086	[illegible]
CsCl	0.120	105	0.080	76
(Ch)Cl	0.105	80	0.078	71

TABLE IV The rate of sodium influx at various levels of extracellular sodium and potassium All the experiments were carried out with cells from Donor J F (pH 7.40, 38° C) The rate of sodium influx was not significantly increased until extracellular potassium concentration exceeded 55 meq/l

Medium	Date	Extracellular sodium (meq/l)	Extracellular potassium (meq/l)	Sodium influx (rate constant) k_1
Plasma	17.4.64	131	5.7	0.056
Plasma	27.5.64	137	4.7	0.056
Chloride	3.8.65	142	5.7	0.058
Ringer's solution	1.9.65	142	5.7	0.059
—	8.11.67	142	20	0.052
—	2.2.68	106.5	55	0.060
—	—	71	91	0.066
—	—	35.5	126.5	0.094
—	—	0.7	162	0.145
—	—	0.4	162	0.157

teristic for large monovalent anions like thiocyanate and salicylate (Wieth 1970 a)

The rate constants of sodium influx found in the KCl, RbCl CsCl and choline chloride media (Table III) showed a much wider range of interindividual variation than was found in similar experiments where the anionic composition of the medium was varied (Funder and Wieth 1967 b) The greatest variations were found in the KCl medium Therefore repeated determinations were performed with two months' interval on cells from two donors in order to evaluate the intraindividual variability This was found to be small the rate constant of sodium influx being 0.084 and 0.094 in the cells of one donor 0.157 and 0.145 in the other donor's cells The same two

lithium than on sodium influx. Accumulation of ^{137}Cs in red cells was linear with time for at least 8 hrs. A cesium influx of 4.7 meq/kg solids and hour was found in the 142 mM CsCl medium to be compared with 3.3 meq/kg solids in the presence of 142 mM $CsHCO_3$.

Discussion

Effects of monovalent cations on Na^+ permeability

The present study was carried out to gather more information about the effect of ions on the permeability of the erythrocyte membrane. The investigation was prompted by the suggestion of Robinson and Jencks (1965) that effects of salts on water structure may be ruled out if the effects caused by a lyotropic series of anions are not mimicked by an analogous cation series.

Water is a bulk component of the cell membrane and must, therefore, figure importantly in the molecular organization of the system (Fernandez-Moran 1962, Berendsen 1967). Electron microscopic studies of partially hydrated erythrocyte ghosts have revealed the presence of 10—20 Å microcrystalline structures of the hydrate type (Fernandez-Moran 1962). In agreement with the view that water plays an essential role in membrane structure, Hechter (1965) has presented a modification of the classical paucimolecular membrane model in which protein and lipid are cemented together by layers of water molecules, which are held in a rigid crystalline lattice structure by the polar groups of lipids and proteins. Ion paths include water layers in the membrane, and the structure of water and its change may, therefore, prove important factors for phenomena as permeability and ion specificity (Berendsen 1967).

The lyotropic series of cations employed in this study represents a wide range of variation of the properties responsible for the interference of ions with water structure. A series of measures related to the interaction between solute and solvent are listed in Table VI. The variations of the viscosity coefficient B (Stokes and Mills 1965) and of structure breaking entropy (Frank and Evans 1945) illustrate the effect of the ions on water structure. Lithium and choline are structure formers, whereas the structure breaking properties increase through the series Na, K, Rb, and Cs. All the anions considered in our previous study (Funder and Wieth 1967 b) are structure breakers, and the structure breaking ability increases through the lyotropic series of anions $Cl < Br < NO_3 < I < SCN$. If the effect of salts on cation permeability was mediated through effects on water structure not related to the charge of the ions, variation of cations should have at least as much effect as variation of the anions.

The results obtained in the bicarbonate media were the most conclusive, because the permeability of red cells to sodium was almost independent of the nature of the accompanying cation (Table II). The results obtained in the chloride media were less clear. Sodium permeability was unchanged when NaCl was replaced by LiCl, but was almost doubled when 142 mM NaCl was substituted with the chlorides of potassium, rubidium, cesium or choline. It is of interest to note that sodium perme-

TABLE VI. Ionic radii, viscosity coefficient B and structure breaking entropy of monovalent cations and anions. The values cited were obtained from the following references: a) Stern & Amis (1959) Table 17, *) Robinson & Stokes (1959) p. 125. b) Stokes and Mills (1965) Table 4.1 and 4.3. c) Frank and Evans (1945) Table V. The structure forming effect of tetra alkylammonium compounds is discussed by Frank and Wen (1957) p. 136.

	Ionic radii a) (Å)		Viscosity coefficient B b)	Structure breaking entropy (ΔS^{st}) c) (e.u.)
	crystallographic	hydrated		
Li^+	0.70	3.4	0.15	−1.1
Na^+	0.97	2.8	0.09	4.0
K^+	1.33	2.3	−0.01	12.0
Rb^+	1.48	2.3	−0.04	14.1
Cs^+	1.67	2.3	−0.05	15.7
Choline	~3.5*	~3.5*	0.12	—
Cl^-	1.81	2.1	−0.01	10.2
Br^-	1.96	2.3	−0.03	13.9
NO_3^-	2—2.5	—	−0.05	19.4
I^-	2.20	2.3	−0.08	17.9

ability was increased almost to the same degree in the presence of the last four cations in spite of the fact that the effect of choline on water structure is essentially different from the effect of potassium, rubidium and cesium (Frank and Wen 1957). Choline is a structure former as reflected by the positive viscosity coefficient B (Table VI). It can therefore be excluded that the effects of the lyotropic series of cations on red cell permeability is due to a non specific structure breaking effect of the ions on a rigid water lattice as suggested by the membrane model of Hechter (1965).

The results of the present work showed several important differences between the effects of cations and anions on the ion permeability of human red cells. The cations do not induce an increased sodium permeability at 0° C comparable to that found in the presence of foreign anions (Wieth 1970 a). Replacement of sodium by potassium had no effect on sulfate permeability, whereas the substitution of chloride by other monovalent anions reduced sulfate permeability considerably (Wieth 1970 b). The increased sodium permeability that accompanies substitution of chloride with thiocyanate or bicarbonate displayed a linear relation to the extracellular anion concentration (Funder and Wieth 1967 b), whereas sodium permeability was not significantly increased until more than 90 meq Na had been replaced by K, Rb or Cs (Table IV). Glynn (1956) and Garrahan and Glynn (1967) have shown that a similar non linear relationship exists between sodium permeability and extracellular choline concentration. Maybe the reduction of sodium concentration rather than the presence of foreign cations is important for the change in sodium permeability.

The object of the present investigation was to investigate whether the permeability changes found in the presence of various anions might be ascribed to physicochemical properties shared by an- and cations. The results show that this is not the case. Evidence suggesting that the anion induced permeability changes are caused by electrostatic binding of adsorbable anions to fixed positive charges in the red cell membrane, is presented elsewhere (Wieth 1970 b).

The effect of bicarbonate on cation permeability

From previous investigation it is known that bicarbonate increases the ability of the red cell membrane to distinguish between Na^+ and K^+ (Funder and Wieth 1967 b). Therefore, it was an interesting observation that the permeability to lithium is increased even more than sodium permeability, when red cells are suspended in a bicarbonate medium at a pH of 7.40 (Fig. 1). From the data of Maizels and Remington (1959) it may be calculated that the initial rate of lithium net flux in a 0.15 M bicarbonate free LiCl medium is about 4 meq lithium/kg cell solids/hr, i.e. one fifteenth of the net flux found in a 162 mM bicarbonate medium (Fig. 1).

Bicarbonate apparently conveys a high selective permeability to small monovalent cations which have a large hydrated radius (lithium and sodium), whereas the permeability to slightly hydrated cations like potassium and cesium is reduced. In the list of specificity patterns of anionic field strength governed cationic selectivity, this cation selectivity is of the type XI (Eisenman 1961). One of the possibilities which must be considered is that the effects found in the presence of bicarbonate is due to an unmasking of fixed anionic charges, which exert the high field strength necessary to produce the specificity pattern mentioned above. It is possible that fixed negative charges with sufficiently high field strength are exposed through an interaction between Ca^{++} and CO_3^{--} in the membrane as discussed by Funder and Wieth (1967 b). However, the bicarbonate effect is not necessarily an effect on the properties of the membrane. The increased influx of Li^+ and Na^+ may in fact be due to ion association between cation and the carbonate ion. Ion pair formation between Na^+ and CO_3^{--} has been suggested by Siggaard Andersen (1962) and in attempt to explain the pH dependence of the pK_2 of carbonic acid, but so far ionpairing of carbonates has otherwise escaped investigation. It might be expected that ion association is at a minimum in the salts of the alkali metals, and this is generally true. However, it is known (Davies 1962) that lithium and sodium hydroxides show clear evidence of ionpairing, although potassium, rubidium and cesium hydroxides are completely dissociated. Lithium and sodium show the same relative affinities for other anions that are proton acceptors as they do for the hydroxyl ion (Robinson and Stokes 1959, p. 424), so it cannot be excluded that they traverse the cell membrane as $LiCO_3^-$ and $NaCO_3^-$. As the permeability to small monovalent anions exceeds cation permeability by a factor of 10^6 (Tosteson 1959), it is obvious that influx of a cation might be increased markedly even at low concentration of the carbonate ion pair. Assuming that the permselective red cell membrane prefers $NaCO_3^-$ to Na^+ by a factor of 10^6, a concentration of $3 \cdot 10^{-6}$ M $NaCO_3^-$ would suffice to treble sodium

influx As the carbonate concentration at a pH of 7·40 is about 6 10⁻⁴ M in the bicarbonate medium, less than 0 5 per cent of the carbonate needs to be present as ion pair to agree with an ion pair hypothesis

A situation in which a cation traverses the red cell membrane in the disguise of an anion has been described by Gray and Sterling (1950) who showed that the Cr^{+++} ion is effectively excluded, whereas CrO_4^{--} permeates the red cell readily The special feature about the carbonate system serving as a carrier for lithium and sodium ions (=X) would be the ability of carbonate to cause a net flux of cations into the cell Because the intracellular concentrations of hydrogen ions and of X are respectively higher and lower than the corresponding extracellular concentrations XCO_3^- reaching the intracellular phase will dissociate according to the reaction $XCO_3^- + H^+ \rightleftharpoons X^+ + HCO_3^-$ and the cation is trapped in the cell The sensitivity of the bicarbonate/carbonate equilibrium to changes of hydrogen ion concentration implicates that the sodium influx should be sensitive to changes of extracellular pH This was in fact noted by Wieth and Funder (1965) who reported an increase of sodium influx of 80 per cent, when extracellular pH in a 142 mM HCO_3^- medium was increased from 7 10 to 7 70

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References

BERENDSEN H J C Water Structure in Theoretical and experimental biophysics (A Cole ed) New York Marcel Dekker Inc 1967 1 1—76

DAVIES C W Ion Association London Butterworths 1962 p 77

DAVSON H The influence of the lyotropic series of anions on cation permeability Biochem J 1940 34 917—925

EISENMAN G On the elementary atomic origin of equilibrium ionic specificity, in Membrane Transport and Metabolism (A Kleinzeller & A Kotyk ed) London Academic Press 1961 163 179

FERNANDEZ MORAN H Cell membrane ultrastructure Low temperature electron microscopy and X ray diffraction studies of lipoprotein components in lamellar systems Circulation 1962 26 1030—1065

FRANK H S and M W EVANS Free volume and entropy in condensed systems III Entropy in binary liquid mixtures partial molal entropy in dilute solutions structure and thermodynamics in aqueous electrolytes J chem Phys 1945 13 507—532

FRANK H S and W Y WEN Structural aspects of ion solvent interaction in aqueous solutions a suggested picture of water structure Disc Faraday Soc 1957 24 133—140

FUNDER J and J O WIETH Determination of sodium potassium and water in human red blood cells Scand J clin Lab Invest 1966 18 151—166

FUNDER J and J O WIETH Effect of ouabain on glucose metabolism and on fluxes of sodium and potassium of human blood cells Acta physiol scand 1967 a 71 113—124

FUNDER J and J O WIETH Effects of some monovalent anions on fluxes of Na and K and on glucose metabolism of ouabain treated human red cells Acta physiol scand 1967 b 71 168—185

GRAY, S J and K STERLING The tagging of red cells and plasma proteins with radioactive chromium J clin Invest 1950 29 1604—1613

GARRAHAN, P J and I M GLYNN The behaviour of the sodium pump in red cells in the absence of external potassium J Physiol (Lond) 1967 192 159—174

GLYNN I M Sodium and potassium movements in human red cells J Physiol (Lond) 1956 134 278—310

HECHTER O Role of water structure in the molecular organization of cell membranes *Fed Proc* 1965 *24* Suppl 15 S 91—S 102

HIPPEL, P H O and K Y WONG, Neutral salts the generality of their effects on the stability of macromolecular conformation *Science* 1964 *145* 577—580

MAIZELS M and M REMINGTON, Cation exchanges of human erythrocytes *J Physiol* (Lond) 1959 *145* 641—657

ROBINSON D R and W P JENCKS, The effect of concentrated salt solutions on the activity coefficient of acetyltetraglycine ethyl ester *J Amer chem Soc* 1965 *87* 2470—2479

ROBINSON, R. A and R H STOKES, *Electrolyte solutions* London Butterworths 1959 p 125 and p 424

SACHS J R. and L. G WELT, The concentration dependence of active potassium transport in the human red blood cell *J clin Invest* 1967 *46* 65—76

SIGGAARD-ANDERSEN, O , The first dissociation exponent of carbonic acid as a function of pH *J clin Lab Invest* 1962 *14* 587—597

SINGER I and I TASAKI, *Nerve excitability and membrane macromolecules,* in Biological Membranes, (D Chapman ed) London, Academic Press 1968 pp 374—410

STERN K H and [illegible] [illegible] AMIS Ionic size *Chem Rev* 1959 *59* 1—64

STOKES, R H and R MILLS *Viscosity of electrolytes and related properties* Pergamon Press Oxford 1965 pp 151

TOSTESON D C , Halide transport in red blood cells *Acta physiol scand* 1959 *46* 19—41

WIETH J O , Effects of bicarbonate and thiocyanate on fluxes of Na and K, and on glucose metabolism of actively transporting human red cells *Acta physiol scand* 1969 *75* 313—329

WIETH J O , Paradoxical temperature dependence of Na and K fluxes in human red cells *J Physiol* (Lond.) 1970 a. In press

WIETH J O , Effect of some monovalent anions on chloride and sulfate permeability of human red cells *J Physiol* (Lond) 1970 b In press

WIETH, J O and J FUNDER An effect of anions on transfer of sodium through the human red cell membrane *Scand J clin Lab Invest* 1965 *17* 399—400

Acta physiol. scand. 1970. 79. 88—94

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Reformation of Taste Buds by Crossed Sensory Nerves in the Rat's Tongue

By

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Abstract

OAKLEY, B. *Reformation of taste buds by crossed sensory nerves in the rat's tongue.* Acta physiol. scand. 1970. 79. 88—94.

Rat taste buds degenerate and disappear when their nerve supply is cut. Regeneration of the taste nerve fibres causes certain epithelial cells to differentiate into slender receptor cells comprising the taste bud. In this study by cross-union the glossopharyngeal (IXth) nerve was made to innervate the front of the tongue, and in other rats the chorda tympani nerve was forced to grow to the back of the tongue. These cross-regenerated sensory nerves were able to cause differentiation of epithelial cells into taste buds, whose number and distribution were histologically determined 15 weeks postoperatively. The cross-innervating IXth nerve reestablished taste buds in the anterior fungiform papillae in conformity with the normal number and distribution of taste buds at the front of the tongue. The chorda tympani, forced to innervate the back of the tongue, reestablished in the foliate papillae more than 1.5 times as many taste buds as it normally innervates. The number of taste buds normally innervated by the chorda tympani must, therefore, be restricted by the capacity of the anterior tongue tissue to respond to this nerve's influence. This study indicates that the distribution and total number of taste buds in the rat tongue is limited by the inherent nature of the tongue region being innervated (tissue-specific), rather than by the type of nerve supplying the taste buds (nerve-specific).

Taste buds on the rat's tongue are located in three different types of papillae. The fungiform papillae are punctate spots 0.3 mm in diameter, which are distributed over the dorsal surface of the anterior two thirds of the tongue. They are especially numerous near the tip of the tongue. Each of the approximately 90 fungiform papillae found on each side of the tongue contains, with rare exceptions, only one taste bud. All of the fungiform taste buds are innervated by the chorda tympani nerve (Fish, Malone and Richter 1944, Whiteside 1927). On the posterior tongue there is a single midline circumvallate papilla with several hundred taste buds embedded in both the inner and outer walls of the nearly circular trench or furrow surrounding the papilla. About 90 % of these taste buds receive bilateral innervation from both IXth nerves (Guth 1963, Whiteside 1927). The third kind of papillae that have taste buds are the foliate papillae on each side of the posterior tongue. These

are a series of 5—8 closely spaced furrows within which numerous taste buds are embedded

When a taste bud is deprived of its normal nerve supply the taste bud degenerates in 1—2 weeks (Vintschgau 1880 Whiteside 1927, Zelena 1964) If the nerve is able to regenerate it will initiate the formation of a new taste bud from the surrounding epithelial cells Oakley (1967) has previously shown that the IXth nerve can be made to innervate the anterior portion of the rat tongue and the chorda tympani *the posterior portion of the tongue Such cross innervation results in a* changed responsiveness of the nerve to taste stimulation of the tongue as measured by summated action potential discharges from the whole nerve That is the taste responses are characteristic of the tissue innervated (tissue specific) and not of the nerve which happens to innervate the region (nerve specific) On this basis one might also expect in the cross innervated rat tongue that the distribution and number of taste buds would be tissue specific and hence independent of the kind of innervating nerve (chorda tympani or IXth nerve) It was the purpose of this investigation to observe histologically the number and distribution of tongue taste buds reformed under the influence of cross innervation by either the chorda tympani or IXth nerve

Methods

Twelve female Sprague Dawley albino rats having either regenerated or cross regenerated taste nerves were used in this experiment The animals were anesthetized with urethane pentobarbital or nembutal and unilateral end to-end sensory nerve anastomoses were performed In two controls the chorda tympani nerve was cut unilaterally and the central portion of the chorda rejoined to the peripheral portion In two other control animals the chorda was cut and the lingual nerve cut central to the branching out of the chorda then the central portion of the chorda was joined to the peripheral portion of the lingual nerve As experimental animals four rats with cross regenerated IXth nerves were utilized in which the central portion of the IXth nerve was sutured to the peripheral part of the chorda tympani Four cross regenerated chorda tympani rats were also used in which the central part of the chorda was sutured to the peripheral part of the IXth nerve In all operations the degenerating peripheral nerve stump served simply as a guide for the regenerating fibers All operations were unilateral Some of the electrophysiological properties of the cross regenerated nerves have been reported and further methodological details may be found in that paper (Oakley 1967) After an average of fifteen postoperative weeks the tongues were fixed in 10 % formalin and embedded in paraffin Complete 10 μ serial horizontal sections of the foliate and circumvallate papillae were mounted on slides and stained with Heidenhain s iron hematoxylin This stain leaves a small dark spot around the taste pore which facilitates the task of counting numerous taste buds in serial sections (Guth 1963) In the experimental and operated control rats the first 50 sections at the tip were discarded and the next 325 serial sections of the front of the tongue were examined for taste buds In three additional rats the entire anterior part of the tongue was serially sectioned (1200 10 μ sections for each rat) In two of these rats the entire posterior part of the tongue was also examined (700 10 μ sections for each rat) 3 weeks after transection of the IXth nerves

Results

102 83 and 95 taste buds (Mean=93) were counted unilaterally in the 3 rats whose entire anterior tongue was serially sectioned In these and in all other rats examined all taste buds on the anterior portion of the tongue occurred without exception in the fungiform papillae In over 98 % of the fungiform papillae there was only one taste

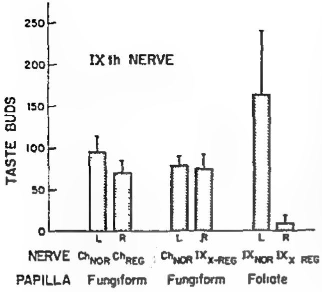


Fig 1 The number of taste buds innervated by the control regenerated chorda tympani and cross regenerated IXth nerves. Each rat served as its own control Thus for each of the three pairs of bars the left (L) or normal side of the tongue is compared with the right (R) or experimental side Each bar is the mean of four values dotted = control rats cross-hatched = experimental rats One standard deviation is shown The legend at the bottom indicates the type of innervating nerve (normal regenerated or cross regenerated) and the location of the taste buds. The counts for the fungiform taste buds were multiplied by 2 1 to obtain the total number of fungiform taste buds

bud per papilla double taste buds or the complete absence of a taste bud in a fungiform papilla occurred less than 2 % of the time The only taste buds found in serial sections of the entire posterior tongue after bilateral IXth nerve section in two of the above rats were located in the foliate papillae Counts of foliate taste buds in six additional rats after IXth nerve section gave a mean of 16 foliate taste buds unilaterally (N = 8 SD = 7) (This is in agreement with Whiteside (1927) who has shown that the chorda tympani nerve innervated a few taste buds in the foliate papillae) Thus the taste buds normally innervated by the chorda tympani nerve comprise an average of 93 fungiform taste buds and 16 foliate taste buds for an average total of 109 taste buds To eliminate both the time consuming examination of the entire anterior part of the tongue and the difficulty of counting taste buds at the extreme tip of the tongue a sampling procedure was developed From the counts of taste buds on the entire anterior part of the tongue in 3 rats and from counts on at least 0 5 cm of seven additional anterior tongues it was found that the number of fungiform taste buds could be reliably determined by examining the front of the tongue 47 % ± 15 % of the fungiform taste buds are contained within a 3 25 mm block of tongue tissue 0 5 to 3 75 mm from the tip of the tongue (Counts of taste buds on the two sides agreed within 10 % which was also the same as the left right variation in counts of fungiform taste buds for the total anterior tongue) Thus in the experimental and operated control rats the status of fungiform taste buds was determined by directly counting all taste buds in this 3 25 mm block of tissue at the front of the tongue

All control regenerated and cross regenerated taste nerves reformed taste buds and these were located in the taste papillae characteristic of the tongue region invaded by the nerve. The ability of control regenerated chorda tympani nerve fibres

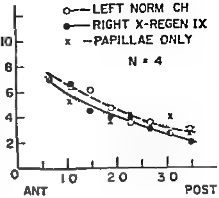


Fig 2 The spatial distribution of fungiform taste buds innervated by normal and cross regenerated nerves Each data point is the mean of four animals and is placed at the mid point of its 400 μ sample of tongue tissue, with sampling beginning 0.5 mm from the tip of the tongue The data for the papillae only (denervated and therefore lacking taste buds), comes from the 4 experimental animals having cross-regenerated chorda tympani nerves which innervated the posterior tongue region Ordinate number of taste buds Abscissa mm

to reestablish fungiform taste buds is shown by the left pair of bars in Fig 1 The normal number of fungiform taste buds on the left side of the tongue is compared with the number of fungiform taste buds reformed on the right side by the regenerated chorda tympani nerve (It made no difference whether the chorda tympani had been sutured to itself for regeneration or whether it had been sutured to the peripheral stump of the lingual nerve)

In 4 experimental rats the cross regenerated IXth nerve successfully reformed single taste buds in the fungiform papillae (center pair of bars in Fig 1) The pair of bars on the right in Fig 1 indicates that very few taste buds remain in the right foliate papillae when the IXth nerve is forced to the front of the tongue One may therefore conclude that the right foliate papillae which were initially denervated by the nerve crossing operation, were not quantitatively resupplied by the IXth nerve growing back from the anterior part of the tongue Since only the left IXth nerve remained to innervate the posterior part of the tongue it was also possible to count the total number of taste buds normally innervated by one IXth nerve in the circum vallate and foliate papillae (Mean = 647 ± S D 135) The failure to observe an abnormally large number of taste buds on the front of the right side of the tongue could have been due in whole or part to inadequate cross regeneration of the taste fibres within the IXth nerve However cross innervation by the IXth nerve seemed to be no less effective than control reinnervation by the chorda tympani in that in both cases 8—10 % of the fungiform papillae lacked taste buds (In normal rats less than 1 % of the fungiform papillae lack taste buds) As shown in Fig 2 the spatial distribution of cross innervated fungiform papillae at the front of the tongue on the right is indistinguishable from the normal distribution of fungiform taste buds on the left side of the tongue A similar spatial distribution of denervated fungiform papillae without taste buds was found as determined by examining the front of the tongue of the four rats with a cross regenerated chorda tympani nerve Thus there was no evidence from an examination of the front of the tongue that the taste buds inner

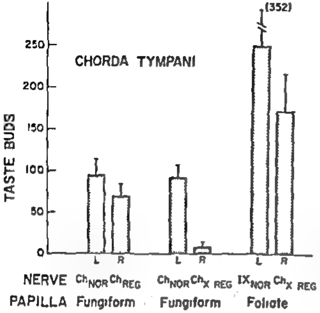


Fig 3 The number of taste buds innervated by the control regenerated and cross regenerated chorda tympani nerves Axes and the left pair of bars as in Fig 1

vated by the cross regenerated IXth nerve were abnormal in their relationship to fungiform papillae abundance or spatial distribution

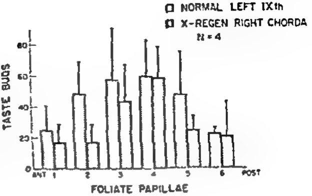
When the chorda tympani is forced to innervate the posterior tongue area most taste buds permanently disappear from the fungiform papillae and numerous taste buds are reformed in the foliate papillae The average number of foliate taste buds innervated by the cross regenerated chorda tympani nerve was almost twice the number of fungiform taste buds innervated by the chorda tympani on the normal side of the tongue (Fig 3 center and right pair of bars) The number of foliate taste buds innervated by the cross regenerated chorda tympani nerve of these four experimental rats was significantly greater than the total number of taste buds (fungiform and foliate) normally innervated by the chorda tympani nerve as determined by serial sectioning of the entire tongue of three control rats ($p = 0.028$ Mann Whitney U test)

The number of furrows in the foliate papillae varied from 5—7 in the 4 experimental rats with a cross regenerated chorda tympani nerve The distribution of taste buds in these furrows is presented in Fig 4 There is no tendency apparent for the cross innervated taste buds to predominate in one furrow they appear to be distributed throughout all furrows in the normal fashion

Discussion

The basic result of this study is that cross regenerated tongue sensory nerves can reform taste buds and their number and distribution closely approximates the normal situation for the particular tongue region That is the location and density of taste

Fig 4 Average number of taste buds for 4 rats occurring in 6 foliate papillae The most anterior furrow on each side was defined as 1, and the more posterior furrows were then numbered consecutively (Only two rats had the 6th papilla Ten taste buds in a 7th papilla of one rat are not shown) One standard deviation is plotted



buds is tissue specific This means that the chorda tympani nerve, for example, which normally innervates an average total of 109 taste buds will if directed to a favorable region (the posterior tongue), form more than one and one half times as many taste buds Accordingly, the limiting factor in the number of taste buds normally innervated by the chorda tympani nerve is the capacity of the anterior tongue tissue to respond to its influences

Because the circumvallate papillae receives innervation from both IXth nerves about 90 % of its buds should have survived the nerve crossing operations (Guth 1963 Whiteside 1927) It is likely that the cross regenerated chorda tympani nerve innervated some of the circumvallate taste buds (with or without mutual innervation by the intact IXth nerve), but this can only be determined quantitatively in experiments which also cut the remaining IXth nerve In the present experiment there was no significant difference between the number of taste buds in normal circumvallate papillae and the number in the circumvallate papillae of the four experimental rats with a cross-regenerated chorda tympani nerve Previous electrophysiological investigation indicated that cross regenerated chorda tympani fibres extended to the circumvallate papilla region (Oakley 1967)

In the normal rat the chorda tympani nerve innervates an average of 16 foliate taste buds and all of these are in the anterior folds Why doesn't it normally innervate more since in cross regeneration it is perfectly capable of forming taste buds in all of the foliate papillae? It is tempting to point to the example of vertebrate skeletal muscle where the initial innervation of a muscle fibre usually excludes further nervous innervation (Harrison 1910) Thus the IXth nerve fibres may arrive first in development and/or successfully dominate in competition with the chorda tympani endings However since multiple innervation is known to exist in the sense of taste (90 % of the taste buds in the circumvallate papilla are bilaterally innervated (Guth 1963 Whiteside 1927)) it is more likely that the chorda tympani normally sends only a few fibres to the region of the foliate papillae and these fibres simply lack the capacity to form or innervate a large number of taste buds

There are three major conclusions of this study

1 Taste buds can be reformed by cross-innervation of the tongue with the IXth or chorda tympani nerves
2 *Both the distribution and number of such cross innervated taste buds are characteristic of the tongue region being innervated (tissue specific)*
3 When placed in a favorable tissue environment (i e, the posterior part of the tongue) the chorda tympani is capable of initiating the formation of more than one and one half times as many taste buds as it normally innervates

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References

FISH H S P D MALONE and C P RICHTER The anatomy of the tongue of the domestic Norway rat I The skin of the tongue the various papillae, their number and distribution *Anat Rec* 1944 *89* 429—440

GUTH L Histological changes following partial denervation of the circumvallate papilla of the rat *Exp Neurol* 1963 *8* 336—349

HARRISSON R G The outgrowth of the nerve fiber as a mode of protoplasmic movement *J Exp Zool* 1910 *9* 787—846

OAKLEY B Altered temperature and taste responses from cross regenerated sensory nerves in the rat s tongue *J Physiol* (Lond) 1967 *188* 353—371

VINTSCHGAU M VON Beobachtungen uber die Veranderungen der Schmeckbecher nach Durchschneidung des n Glossopharyngeus *Pflugers Arch ges Physiol* 1880 *23* 1—13

WHITESIDE B Nerve overlap in the gustatory apparatus of the rat *J comp Neurol* 1927 *44* 363—377

ZELENA J Development degeneration and regeneration of receptor organs In *Progress in Brain Research 13 Mechanisms of Neural Regeneration* pp 175—213 Ed by M Singer and J P Schade Elsevier, Amsterdam 1964

Acta physiol. scand. 1970. 79. 95—108

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Intrarenal Blood Flow and Para-aminohippurate (PAH) Extraction

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Abstract

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Renal hyperemia induced by acetylcholine or bradykinin reduces the renal extraction of p-aminohippurate (E_{PAH}). If PAH is completely extracted from blood passing through the renal cortex and no PAH is extracted from the juxtamedullary circulation, the medullary flow fraction (1-E_{PAH}) should increase during vasodilatation. To test this hypothesis, E_{PAH} was measured in 12 dogs during infusion of acetylcholine and bradykinin into the renal artery; renal blood flow (RBF) was measured by electromagnetic flowmeter, and H_2-clearance from [illegible] (bradykinin), whereas the hypothesis predicted a rise to more than 200 per cent. E_{PAH} is not, therefore, a valid indicator for intrarenal blood flow distribution.

A negative correlation between E_{PAH} and RBF was also observed when RBF was suddenly reduced by renal artery constriction. The results are compatible with flow-dependent cortical extraction and no extraction of PAH from medulla. Renal sodium excretion showed no correlation to medullary blood flow.

Intrarenal distribution of blood flow is commonly assumed to be of considerable importance for the concentrating mechanism and possibly also for the regulation of renal salt excretion. Unfortunately, no simple and reliable method is available for selective measurement of medullary and cortical blood flow in the intact animal. An indirect method, using the renal extraction of p-aminohippuric acid, E_{PAH}, has been proposed by Reubi (1958), and has received some experimental support (Pilkington *et al.* 1965). This method is based on the hypothesis that the cortical fraction of blood flow is totally freed from PAH in one passage, and that no PAH is extracted from the medullary flow fraction. It follows that E_{PAH} represents the fraction of plasma flow to the cortex, and medullary blood flow may be expressed as RBF(1-E_{PAH}), where RBF = total renal blood flow.

An increase in RBF is regularly associated with decreased E_{PAH}. According to the above equation, an increase in RBF from 100 to 200 ml/min, with a decrease in E_{PAH} from 0.80 to 0.70, would mean an increase in medullary blood flow of 200 per cent, and an increase of only 75 per cent in cortical flow. The main purpose of the present study was to evaluate this hypothesis by comparing changes in E_{PAH} induced by vasodilating drugs (acetylcholine and bradykinin) to medullary blood flow as indicated by local clearance of hydrogen gas from the outer medulla (O.M.H-clearance). It was found that both drugs reduced E_{PAH}, despite a reduction in the medullary flow fraction, which means that E_{PAH} is not solely determined by cortico-medullary flow distribution. Instead, the results suggest incomplete and flow dependent extraction from cortical blood. If this were the case, PAH extraction should increase with reduced renal blood flow. Since a severe fall in GFR and urine flow will sooner or later reduce and eventually stop PAH extraction, measurements were made immediately after reducing renal blood flow by constriction of the renal artery. A transient or lasting elevation of E_{PAH} was invariably observed.

The alleged dependence of renal sodium excretion on medullary blood flow was studied by comparing changes in sodium excretion and outer medullary hydrogen clearance during vasodilatation. No significant correlation, positive or negative, was observed.

Material and methods

12 *dogs of both sexes (mean weight 19.4 kg) were used for investigating the effect of acetyl*choline and bradykinin on renal medullary blood flow and PAH extraction. The animals were fasted for 18 hrs before an experiment but had free access to water. They were anesthetized with Nembutal 25 mg/kg i.v. initially, and received subsequent doses of 1—2 mg/kg when necessary to maintain light anesthesia throughout the experiment. A tracheal cannula was inserted to secure free airways. A femoral artery was catheterized for pressure recording and blood sampling.

Priming and sustaining solutions of creatinine and sodium para aminohippurate (in saline of 0.9 % 8 experiments or 0.45 % 4 experiments) were administered i.v. through a catheter in a femoral or brachial vein to give constant plasma concentrations of 1.0 to 2.0 mg % for PAH and about 15 mg % for creatinine. The infusion rate was varied from 2—5 ml/min but was kept constant during each experiment. A subcostal incision was made for retroperitoneal approach to the right or left kidney. The renal artery and vein were freed from surrounding tissue, care being taken to avoid damage to renal nerves. An electromagnetic flow probe was applied to the artery and a polyvinyl tube of 0.4 mm external diameter was inserted into the artery in retrograde direction *ad modum* Herd and Barger (1964). A catheter was guided *via the femoral vein into the renal ve* [illegible]
inserted from the lateral aspect of the [illegible] th
of the tapered electrode tip was 1.0— [illegible] ed
electrode shaft was stitched to the cap [illegible] d
the wound temporarily closed.

Local hydrogen concentration was measured polarographically with a 6-channel polarograph and recorded on a Rikadenki 6-channel recorder (Rikadenki Co. Tokyo, model B64) using a polarizing potential versus a KCl-saturated calomel reference electrode of +0.2 V (Aukland, Bower and Berliner 1964, Aukland 1968). Hydrogen gas was supplied by inhalation of about 5 % H_2 in air for 3—10 min, providing a stable concentration in the outer medulla. After [illegible] for another 1—2 min by infusion of [illegible] desaturation *of renal arterial* blood [illegible] drogen clearance per volume tissue [illegible] ant (k min^{-1}) of the desaturation [illegible] on of each electrode was checked [illegible] m deep in the medulla, measured on the excised [illegible]

from the cortico-medullary border and not in close contact with calyces or macroscopical blood vessels Erroneous positions of the electrodes could usually be predicted from the desaturation curves but their position checked at the end of each experiment was the criterion for exclusion

Renal venous blood samples were immediately cooled in ice water and centrifuged at 4° C to minimize PAH diffusion from erythrocytes into plasma Creatinine was measured by the method of Bonsnes and Taussky (1945) and PAH according to Smith *et al* (1945) Renal blood flow (RBF) was measured with a Nycotron electromagnetic flowmeter, type 372 (Nycotron Oslo), and recorded continuously on a Sanborn recorder Calibration of the flowmeter probes (2 5 mm or 3 mm) had been performed directly on femoral or carotid arteries of comparable size This calibration usually agreed well with renal blood flow estimated from PAH clearance, extraction and hematocrit Arterial blood pressure was measured with a Statham transducer and recorded continuously on a Sanborn recorder

Experimental procedure Measurements were started 1/2—1 hr after the end of the surgical procedure and were performed during steady RBF and urine flow O M H_2-clearance GFR RBF and E_{PAH} were measured under control conditions and during i a infusion of acetylcholine or bradykinin alternately, blood and urine samples being collected during hydrogen desaturation For comparison of different infusion periods O M H_2-clearance for each electrode and RBF were calculated in per cent of the mean pre and post infusion control values Average O M H_2-clearance was then calculated for all accepted electrode sites Measurements differing by more than 20 per cent in the two control periods were excluded Acetylcholine was given by constant infusion into the renal artery at rates of 1—18 μg/min in 19 periods in 10 dogs Bradykinin was infused at rates of 0 25—3 2 μg/min in 16 periods in 9 dogs

To block cholinesterase activity physostigmine was administered in two dogs as a priming dose of 1 mg into the renal artery followed by 3—4 μg/min i v Phenoxybenzamine 1 mg/kg was given to 2 dogs into the renal artery to block a possible effect of circulating or locally

renal artery was con of control and kept pling of arterial and renal venous blood was started a few seconds after flow reduction and continued for 3—5 min

Since local hydrogen clearance is determined by blood flow per volume tissue any change in volume will influence measured clearance In three dogs therefore, changes in outer medullary volume during acetylcholine and bradykinin infusion were estimated by ultrasonic measurement of intrarenal distances Piezoelectric crystals 1 x 2 mm were implanted in the outer medulla, 6—8 mm apart and the distances recorded as will be described in more detail in another communication from this laboratory (Johannesen *et al* in preparation) Dimensions were measured both radially (thickness of outer medulla and parallel to the kidney surface (longitudinally) in the middle of the outer medulla

Results

A protocol of a typical experiment is shown in Table I Control RBF averaged 3 12 ml/min g and E_{PAH} 0 77 During two infusion periods acetylcholine increased RBF to 176 per cent and 186 per cent of control respectively, and bradykinin to 177 per cent and 165 per cent E_{PAH} fell both during acetylcholine and bradykinin infusion to such an extent that the Reubi hypothesis (medullary flow = RBF(1 E_{PAH})) would predict a rise in medullary blood flow of more than 100 per cent in all infusion periods As evident from Table I the observed rise in O M H_2 clearance was much smaller, especially with acetylcholine infusion, which increased O M H_2 clearance by a maximum of 15 per cent

Qualitatively similar results were obtained in all experiments but the effect of both acetylcholine and bradykinin on O M H_2 clearance varied considerably between animals However in the same kidney different electrodes showed good agreement and the response to the vasodilators was usually well reproducible The results of all

TABLE I Protocol of one experiment

Time min	Experimental condition	RBF ml/min	%	Outer medullary hydrogen H_1 ml min^{-1}	%	H_2 ml min^{-1}	%
0	Control	125		28		34	
29	Acetylcholine 4 0 μg/min	229	176	36	—	41	110
54	Control	135		36		37	
76	Bradykinin 0 8—1 2 μg/min	239	177	50	145	45	19
98	Control	135		33		39	
121	Acetylcholine 8 0 μg/min	270	190	40	111	46	112
149	Control	150		39		43	
168	Bradykinin 1 2—1 6 μg/min	239	165	55	147	60	159
186	Control	135		36		40	

RBF = renal blood flow El H1, H2 and H3 = individual electrode measurements F_{PAH} = ex

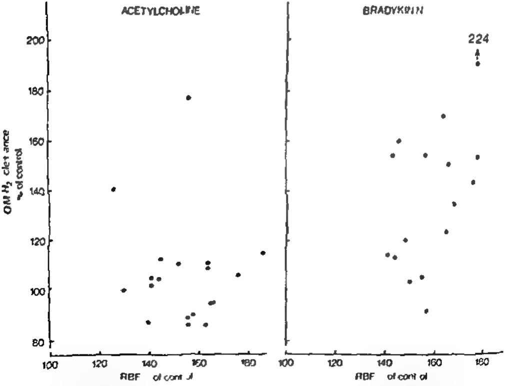


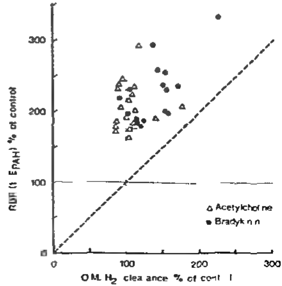
Fig 1 Outer medullary hydrogen clearance (O M H_2 clearance) averaged for each experimental period related to total renal blood flow (RBF) Both in per cent of control

clearance

El, min⁻¹	%	El, min⁻¹	%	Average El, %	E_{PAH}	RBF(1—E_{PAH}) ml/min	%	V ml/min	C_{Cr} ml/min
.29		40			79	26		33	23
31	103	40	100	106	71	66	228	92	26
31		40			76	32		47	22
39	135	66	167	142	66	81	258	71	23
27		39			77	31		61	22
40	—	50	122	115	62	97	277	1 32	24
43		43			75	39		1 40	29
51	131	67	162	150	64	86	236	1 50	25
35		40			75	34		1 28	22

traction of p-aminohippurate V = urine flow C_{Cr} = creatinine clearance.

experiments are summarized in Fig 1 O M H_2-clearance average of all electrodes in each period) during acetylcholine and bradykinin infusion is plotted against total renal blood flow, both in per cent of control values as described above With few exceptions, the percentual increase in O M H_2-clearance was less than the increase in total renal blood flow, the discrepancy being most pronounced during acetyl choline infusion On average, acetylcholine increased total renal blood flow to 154 per cent and medullary blood flow to only 106 per cent of control Corresponding values with bradykinin were 158 per cent and 138 per cent



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aged for each experimental period both in per cent of control

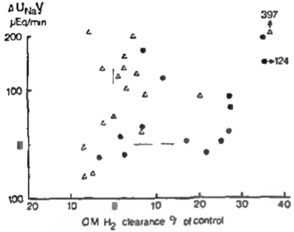


Fig 3

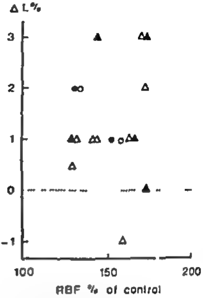


Fig 4

Fig 3 Lack of correlation between changes in outer medullary hydrogen clearance (O.M. H_2-clearance) and renal sodium excretion ($U_{Na}V$) during acetylcholine (triangles) and bradykinin (circles) infusion

Fig 4 Longitudinal (filled symbols) and radial (open symbols) distances in outer medulla in per cent of control ($\Delta L\%$) during acetylcholine (triangles) and bradykinin (circles) infusion related to renal blood flow (RBF) in per cent of control

On average E_{PAH} was reduced from 0.794 to 0.724 by acetylcholine and from 0.788 to 0.698 by bradykinin. Infusion of physostigmine or phenoxybenzamine, each in two experiments did not alter the effect of acetylcholine or bradykinin on RBF, O.M. H_2-clearance or E_{PAH}. The results from these experiments are therefore included in the present data.

A comparison between percentual changes in medullary blood flow calculated from the formula MBF = RBF(1-E_{PAH}) and the observed changes in O.M. H_2-clearance is shown in Fig 2. The rise in O.M.H. clearance representing medullary blood flow was smaller than the rise calculated according to Reubi in every experimental period. In other words the observed rise in medullary blood flow was insufficient to account for the fall in E_{PAH}.

Urine flow averaging 0.60 ml/min in control periods rose in most infusion periods (average +0.72 ml/min) with no consistent difference between acetylcholine and bradykinin. Sodium excretion averaged 96 μeq/min in control periods and rose by an average of 89 μeq/min during infusions. As evident from Fig 3 the rise in sodium excretion ($\Delta U_{Na}V$) showed no correlation to the rise in O.M.H_2 clearance during vasodilatation. In fact sodium excretion rose on average more during acetylcholine infusion (+106 μeq/min) than with bradykinin (+63μeq/min), in spite of smaller increase in O.M.H_2 clearance (Fig 1).

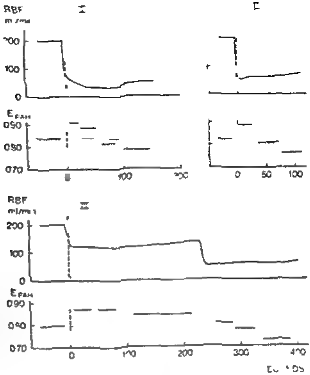
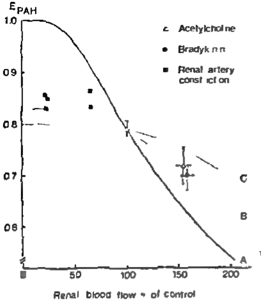


Fig 5 Effect of sudden reduction of total renal blood flow (RBF) on the extraction of p aminohippurate (E_{PAH}) at three consecutive constrictions of the renal artery in one dog

Glomerular filtration rate (GFR) averaged 33 ml/min in control periods and increased slightly during vasodilatation (-4 to $+11$ ml/min, average $+2.4$ ml/min) Changes in sodium excretion were significantly correlated to changes in GFR $\Delta U_{Na}V = 11.4 \cdot \Delta GFR + 61.6$ ($r = 0.52$, $p < 0.05$ $\Delta U_{Na}V$ in μeq/min GFR in ml/min)

The influence of acetylcholine and bradykinin infusion on the distance between two piezoelectric crystals implanted in the outer medulla is shown in Fig 4 The distance usually increased, and seemed better correlated to urine flow than to blood flow No obvious difference was observed between the two drugs nor between longitudinal and radial dimensions The greatest linear increase was observed with acetylcholine and amounted to 3 per cent of control indicating a maximum volume expansion of about 10 per cent

Acute reduction of RBF induced by constriction of the renal artery invariably caused a temporary or lasting rise in E_{PAH} As exemplified in Fig 5 E_{PAH} rose considerably above control during the first minute of flow reduction from 200 ml/min to 40—50 ml/min and then fell below control level (Fig 5 I II) At a more moderate flow reduction — to 130 ml/min — the increase in PAH lasted longer and was still above control after 200 sec (Fig 5 III) Further reduction of flow to 60 ml/min caused a rapid fall in E_{PAH} Experiments in another dog gave similar results



(E_{PAH}) per cent
A B C

and the maximum extraction observed in these experiments corrected to a control extraction of 0.79 for comparison with acetylcholine and bradykinin data have been plotted against RBF in Fig. 6.

Discussion

The hypothesis that E_{PAH} is determined by the distribution of blood flow stems from the following considerations. PAH is extracted only by the proximal convoluted tubules. Blood flowing through the juxtamedullary glomeruli is delivered directly to the medullary vasa recta without contact with proximal tubules and should therefore not be extracted. Although this deduction from the anatomical arrangement may be questioned (Maxwell, Breed and Smith 1950), recent experiments of Slotkoff, Eisner and Lilienfield (1968) strongly suggest that there is little or no extraction from the medullary blood. However, the use of E_{PAH} for estimating corticomedullary flow distribution further demands that the extraction from blood flowing through the cortical peritubular capillaries is 100 per cent. The present results indicate that one or both of these assumptions are invalid. Infusion of acetylcholine and bradykinin in all instances led to increased total renal blood flow and reduction of E_{PAH}. The Reubi hypothesis would therefore demand that medullary blood flow should increase out of proportion to total renal blood flow. Our experiments, however, showed that local clearance of hydrogen gas from the outer medulla increased less, on average, than total renal blood flow. This was especially the case during acetylcholine infusion (Fig. 1), but even during bradykinin infusion the increase was never sufficient — according to the Reubi hypothesis — to account for

the fall in E_{PAH} (Fig 2) The hypothesis is therefore not valid if O M H_2-clearance is a reliable indicator of medullary blood flow Previous studies have provided considerable evidence that this is the case the influence of factors other than blood flow on hydrogen clearance from the outer medulla has been shown to be of minor importance (Aukland and Berliner 1964, Aukland 1967) The active electrode tip is more than 1 mm long and placed at right angles to the vasa recta bundles It is therefore likely that the observed washout rate is determined by blood flow both in the bundles and in the peritubular capillary network, so that any change in total *medullary blood flow should be reflected by changes in the clearance from the outer* medulla Furthermore, the demonstration of autoregulation of medullary blood flow by this method strongly suggests that the normal regulation of local blood flow is not abolished by insertion of the electrodes (Aukland 1967) The close agreement with other methods (Aukland and Wolgast 1968) also shows that O M H_2-clearance is a reliable indicator for medullary blood flow It should be noted, however that the clearance is measured per volume of tissue (ml/min • ml) Swelling of the tissue will therefore reduce the measured clearance relative to total blood flow through the medulla Measurements of intrarenal distances in the outer medulla during vasodilatation however, *showed that volume expansion was less than 10 per cent* even during the highest RBF increase As evident from Fig 1 an upward correction by 10 per cent of medullary flow would lead to approximately equal average increase of medullary and total renal blood flow during bradykinin infusion, while the rise in medullary flow during acetylcholine infusion would still be much smaller than the increase in total renal blood flow In no case would the corrected values satisfy the hypothesis that medullary blood flow equals RBF(1 E_{PAH}) and it is therefore pertinent to discuss mechanisms other than cortex/medulla flow distribution as determinants of renal PAH extraction

The fall in E_{PAH} *during vasodilatation induced by* acetylcholine *and* bradykinin might be due to one or more of the following mechanisms

1 Inhibition of cellular PAH transport by the two drugs This possibility has not been excluded but it seems unlikely that two so different drugs would inhibit the transport system to the same extent It should also be noted that absolute PAH excretion increased in all infusion periods

2 Opening of arteriovenous shunts To our knowledge no direct evidence for shunting of the required magnitude has been presented

3 Inproportionate increase of blood flow through non-extracting areas of the kidney Although the present data do not rule out the assumption of no PAH extraction from medullary blood the fall in extraction during vasodilatation was evidently not due to inproportionate rise in medullary blood flow and it is also difficult to imagine that other areas of the kidney could serve this role

4 Blood flow dependent extraction

a) E_{PAH} could be determined by capillary transit time and PAH-concentration according to Michaelis Menten kinetics as proposed by Kiil (1961) At low plasma concentrations of PAH this hypothesis predicts practically the same dependency on

blood flow as diffusion limited transport, as will be considered in more detail in the following.

b) Diffusion limited transport. PAH transport from plasma to the tubular lumen must begin with diffusion from peritubular capillaries to some point at or within the proximal tubular cells, where the concentration may be assumed to be zero. Complete extraction therefore depends on a negligible concentration gradient at the end of each capillary. Increasing blood flow reduces the time available for PAH diffusion from a given blood element, tending to raise the diffusion gradient and thereby lower the extraction. PAH transport per unit time will still rise with increasing blood flow, but less than proportional to flow, and is therefore said to be "diffusion limited." Since the uptake of other ions from blood, e.g., rubidium in the myocardium, is clearly diffusion limited (Love and O'Meallie 1963), it would be surprising not to find a similar relationship for PAH transport in the kidney with its very high blood flow. With diffusion limited transport, the extraction (E) falls with increasing blood flow (F), according to the equation

$$E = 1 - e^{-\frac{P}{F}}$$

(Renkin 1955). The constant P may be chosen to give an extraction of 0.79 at control flow, as found in the present experiments, resulting in curve A, Fig. 6. According to this curve, a flow rise to 160 per cent of control should reduce E_{PAH} to about 0.60. The observed reduction was less in all infusion periods, indicating that the PAH extraction is not determined by diffusion limited transport alone. Nevertheless, if supplemented with the assumption of a nonextracting area, diffusion limited PAH transport might still explain the fall in E_{PAH} during vasodilatation.

As a first approximation, we assume that nonextracting tissue receives a constant fraction of flow at all levels of RBF, and that the extraction from the remaining flow fraction is determined by the equation shown above. With a fraction of 17 per cent perfusing nonextracting tissue, the extraction from the remaining 83 per cent of blood has to be 0.95 to give the observed overall extraction of 0.79 at control flow. The constant P is thereby determined (2.813), resulting in curve B, Fig. 6, which fits well to the observed data. (Curve C was obtained by choosing a fraction of 20 per cent perfusing nonextracting tissue.)

The question then arises whether the uncleared fraction could be represented by medullary blood flow, as suggested by the observation of Slotkoff *et al.* (1968) that PAH is not accumulated in the renal medulla in the absence of tubular flow. *A priori* this might seem unlikely, since the observed fall in E_{PAH} was not very different with acetylcholine and bradykinin, in spite of the different response of medullary blood flow. However, the following calculations indicate that the data are well compatible with medulla as a source of uncleared blood. As shown above, a control medullary flow (MF) of 17 per cent of RBF and cortical extraction (E_c) of 0.95 results in a total extraction of 0.79, as observed in control periods. From these premises and the observed average changes in RBF (+54 and +58 per cent) and medullary blood flow (+6 and +38 per cent) during acetylcholine and bradykinin infusion, the

resulting E_{PAH} can be calculated For brevity, the calculation will only be carried out for the acetylcholine data with the results for bradykinin added in brackets

Medullary flow fraction during acetylcholine infusion (Ach)

$$\left(\frac{MF}{RBF}\right)_{Ach} = 0\ 17\ \frac{106}{154} = 0\ 117\ (0\ 148)$$

Cortical flow fraction

$$\left(\frac{CF}{RBF}\right)_{Ach} = 1 - 0\ 117 - 0\ 883\ (0\ 852)$$

Cortical flow during Ach relative to control

$$CF_{Ach}/CF_0 = 1\ 54\ \frac{0\ 883}{0\ 83} = 1\ 64\ (1\ 62)$$

Cortical extraction during Ach

$$(E_C)_{Ach} = 1 - e\ \left(\frac{2\ 813}{1\ 64}\right) = 0\ 82\ (0\ 82)$$

Total renal extraction during Ach

$$(E_{PAH})_{Ach} = 0\ 82\ \ 0\ 883 = 0\ 723\ (0\ 700)$$

These figures correspond well to the observed average extractions of 0 724 and 0 698 during acetylcholine and bradykinin respectively Correction of the observed changes in medullary blood flow for 10 per cent tissue swelling changes the calculated extractions by less than 0 01, and does not therefore invalidate the conclusion that the data are well compatible with no PAH extraction in the medulla and flow dependent extraction from cortical blood Possible negative extraction in the medulla due to back leakage of PAH from collecting ducts to vasa recta (Schnermann and Thurau 1965) would obviously modify the estimate of medullary flow fraction but would not in principle contradict the present interpretation

The assumed medullary flow fraction of 0 17 and corresponding cortical extraction of 0 95 under control conditions represent the best possible fit to the observed values but should be considered with caution because of the considerable scatter around the mean values for E_{PAH} RBF and O M H-clearance However the medullary flow fraction is unlikely to fall beyond the limits of 0 12 and 0 19 with corresponding cortical extractions of 0 975 and 0 90 These figures are reasonably well compatible with various estimates of medullary blood flow (Harsing and Pelley 1965) and agree well with the average extraction of 0 95 in cortical venous blood in cats observed by Nissen (1968)

Diffusion as a limiting factor for PAH extraction was rejected by Kinter and Pappenheimer (1956) because they found similar extraction of PAH and Diodrast in spite of the great difference in molecular weight of these substances This objec

tion might therefore favour the hypothesis of enzyme kinetic limitation (paragraph 4a), but according to our view the data presented by these authors are insufficient to exclude diffusion limitation.

It follows from the hypothesis presented here that during hyperemia the PAH extraction is mainly determined by cortical blood flow, which again approximately parallels total renal blood flow, E_{PAH} would be relatively more sensitive to changes in the cortex/medulla flow distribution at reduced total flow. With unchanged flow distribution, reduction of RBF should increase E_{PAH}, but not toward 100 per cent as assymptote, in good agreement with the results of acute reduction of renal blood flow (Fig. 6). E_{PAH} rose in all experiments, and would seem to extrapolate to about 0.85 at zero flow. The conflicting results obtained in steady state flow reductions by previous investigators (Harth-Kreienberg and Lutz 1960, Pilkington *et al.* 1965) might well be due to a critical fall in GFR and tubular flow rate in a smaller or larger number of the nephrons. While *this* factor should not interfere with the extraction during the first minutes of flow reduction, it is possible that the extra fluid leaving the kidney during the first minute of reduced perfusion pressure (Heimburg and Ochwadt 1961, Ofstad 1965) could influence the measured extraction. However, even if the extra fluid contained no PAH, its volume would have to exceed half the kidney volume to account for the present findings. In a few unpublished experiments we have also noted increased extraction during moderate (20—40 per cent) steady state reduction of RBF by continuous intra arterial infusion of angiotensin or adrenaline. Since both these drugs reduce O.M.H. clearance in proportion to total renal blood flow (Aukland 1968), the rise in E_{PAH} should not be due to redistribution of blood flow. Recently, similar findings with angiotensin were preliminarily reported by Carriere and Friborg (1968).

The present experiments thus lead to the conclusion that the cortex/medulla distribution of renal blood flow cannot be estimated from E_{PAH} as proposed by Reubi, probably because of incomplete and flow dependent cortical extraction. The assumption of no extraction from medullary blood may still be valid.

The slight or absent increase in medullary blood flow during acetylcholine infusion was an unexpected observation, and might be suspected as resulting from the high cholinesterase activity in the medulla (Fourman 1966). However, this mechanism seems unlikely, since acetylcholine did not cause a larger increase in medullary flow in the presence of physostigmine. Since phenoxybenzamine did not influence the response to acetylcholine, the small increase in medullary blood flow would not seem to be due to local release of noradrenaline. It also seems unlikely that extravascular factors, such as increased tissue pressure, would be responsible, since acetylcholine and bradykinin increased urine flow to approximately the same extent. We must therefore conclude that for unknown reasons the juxtamedullary arterioles and/or the arterial vasa recta are less responsive than the cortical arterioles to the vasodilator action of acetylcholine.

It has been assumed by some investigators that an increase in medullary blood flow should increase renal sodium excretion (Elpers and Selkurt 1963, Earley and

Friedler 1965), whereas Barger and Herd (1967) have proposed the opposite effect The lack of correlation between sodium excretion and medullary blood flow in the present experiments lends no support to any of these theories and rather suggests that medullary blood flow is not an important determinant of renal sodium excretion

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References

AUKLAND K Study of renal circulation with inert gas measurements in tissue *Proc 3rd int Congr Nephrol Washington 1966* 1967 *Vol 1* 188—200

AUKLAND K Effect of adrenaline noradrenaline angiotensin and renal nerve stimulation on intrarenal distribution of blood flow in dogs *Acta physiol scand* 1968 *72* 498—509

AUKLAND K and R W BERLINER Renal medullary countercurrent system studied with hydrogen gas *Circulat Res* 1964 *15* 430—442

AUKLAND K B F BOWER and R W BERLINER Measurement of local blood flow with hydrogen gas *Circulat Res* 1964 *14* 164—187

AUKLAND K and M WOLGAST Effect of hemorrhage and retransfusion on intrarenal distribution of blood flow in dogs *J clin Invest* 1968 *47* 488—501

BARGER A C and J A HERD Study of renal circulation in the unanesthetized dog with inert gases external counting *Proc 3rd int Congr Nephrol Washington 1966* 1967 *Vol 1* 174—187

BONSNES R W and H H TAUSSKY On the colorimetric determination of creatinine by the Jaffe reaction *J biol Chem* 1945 *158* 581—591

CARRIERE S and J FRIBORG Renal medullary blood flow measured with Krypton^{85} and PAH extraction during angiotensin infusion *Clin Res* 1968 *16* 364

EARLEY L E and R M FRIEDLER Studies on the mechanism of natriuresis accompanying increased renal blod flow and its role in the renal response to extracellular volume expansion *J clin Invest* 1965 *44* 1857—1865

ELPERS M J and E E SELKURT Effects of albumin infusion on renal function in the dog *Amer J Physiol* 1963 *205* 153—161

FOURMAN J The relation of the site of cholinesterase activity in the kidney to the form and function of the nephron *J Anat* (Lond) 1966 *100* 430—431

HARSING L and K PELLES Die Bestimmung der Nierenmarkdurchblutung auf Grund der Ablagerung und Verteilung von ^{86}Rb *Pflugers Arch ges Physiol* 1965 *285* 302—312

HARTH O W KREIENBERG and J LUTZ Der Einfluss von Hydramie und Nierendurchblutung auf die renale Extraktion der p Aminohippursaure *Pflugers Arch ges Physiol* 1960 *270* 174—183

HEIMBURG P and B OCHWADT Uber Anderungen von Gewicht und venosem Hamatokrit der Niere als Ausdruck transvasaler Flussigkeitsverschiebung *Pflugers Arch ges Physiol* 1961 *273* 62—70

HERD J A and A C BARGER Simplified technique for chronic catheterization of blood vessels *J appl Physiol* 1964 *19* 791—792

KUL F Dynamics of renal proximal tubular secretion *Nature* (Lond) 1961 *189* 927—928

KINTER W B and J R PAPPENHEIMER Renal extraction of PAH and Diodrast I^{131} as a function of arterial red cell concentration *Amer J Physiol* 1956 *185* 391—398

LOVE W D and L P O'MEALLIE Relationship of blood flow and myocardial Rb^{86} clearance in right and left ventricles *Amer J Physiol* 1963 *205* 382—384

MAXWELL M H E S BREED and H W SMITH Significance of the renal juxtamedullary

RENKIN, E. M., Effects of blood flow on diffusion kinetics in isolated perfused hindlegs of cats. *Amer. J. Physiol.* 1955. *183*. 125—136.
REUBI, F., Objections à la théorie de la séparation intrarénale des hématies et du plasma. *Helv. med. Acta* 1958. *25*. 516—523.
SCHNERMANN, J. and K. THURAU, Mikropunktionsversuche zum Verhalten der PAH-Konzentration im Vasa recta-Blut der Goldhamsterniere. *Pflügers Arch. ges. Physiol.* 1965. *283*. 171—181.
SLOTKOFF, L. M., G. M. EISNER and L. S. LILIENFIELD, Functional separation of renal cortical-medullary circulation: significance of Diodrast extraction. *Amer. J. Physiol.* 1968. *214*. 935—941.
SMITH, H. W., N. FINKELSTEIN, L. ALIMINOSA, B. CRAWFORD and M. GRABER, The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J. clin. Invest.* 1945. *24*. 388—404.

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From the Institute of Zoophysiology, University of Uppsala Sweden

In vivo Recording of Contractile Activity of Male Accessory Genital Organs in Rabbits

By

PER MELIN

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Abstract

MELIN, P *In vivo recording of contractile activity of male accessory genital organs in rabbits* Acta physiol scand 1970 *79* 109—113

lower degree of muscular activity, compared with both the proximal part of the organ and with the ductus epididymidis

Few studies have paid any attention to the contractile behaviour *in vivo* of the various parts of the male reproductive organs and its importance for the transport of spermatozoa (review by Risley 1963) Different methods have been used such as the muscle lever kymographic technique, cinematography and microscopic observations The present work describes a method of studying the contractions of the distal part of the ductus epididymidis, the vas deferens and the vesicula seminalis by means of intraluminal pressure measurements In addition a quantitative analysis has been performed of different characteristics of contractile activity

The observations were made in connection with an investigation of neurohypophysial hormones and male genital contractions to be published elsewhere

Material and Methods

Animals

72 male rabbits about 1 year old, weighing 2 6—3 4 kg and of mixed breeds were used The animals had been caged separately and been given the same kind of food at regular times for at least a fortnight prior to the experiments

Operative procedures and recording technique

The rabbits were anesthetized by giving them i v injections of Numal (Roche) (0 5 ml/kg b w) Tracheotomy was generally performed to facilitate respiration

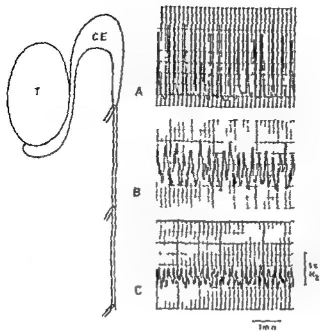

Fig 1 A diagram of the regions of the male genital duct studied and records of their contractile activity The borderlines between the different zones give the approximate positions of the cannula used in pressure measurements
A = ductus epididymidis B = vas deferens proximal part
C = vas deferens distal part CE = cauda epididymidis T = testis

A mid line incision in the abdominal wall was made from the pubic symphysis about 5 cm cranially exposing the internal organs and the bladder was retracted posteriorly emptied and cannulated in order to avoid accumulation of urine To eliminate the disturbing influence on the observed regions of the reproductive organs caused by the passage of faeces the distal part of the colon and rectum were emptied and a ligature was tied around the colon proximally The intestines were retracted cranially and held in position with pads of cotton wool moistoned in warm saline The temperature of the animals was kept at 37° C by means of a homoeothermic blanket control

Ductus epididymidis With one testis in the abdominal position a small incision was made in the wall of the extreme proximal end of the vas deferens and a polythene cannula was introduced into the lumen of the ductus epididymidis and fixed in position with a suture (Fig 1) The cannula was attached by means of a three way valve to a water manometer (for pressure calibration) and the manometer was connected to a transducer (Statham Model P23DB) The system was filled with Tyrode solution after which the junction to the manometer was closed and the intraluminal pressure in the organ was registered by a Varian ink recorder via an amplifier (Tekronix Type 3C66) The pressure changes in this closed system thus reflect under the assumptions given the isometric contractions originating from muscle activities in

a ligature was made
n the wall of the vas
the blind sac of the
vas deferens obtained in this way as earlier described

Vas deferens distal part The exposed vas deferens was ligatured in the middle The cannula was introduced just proximally of the ampullae and the pressure measured as before (Fig 1)

The initial intraluminal pressure was kept at 75 mm H_2O for all the different genital duct preparations

Vesicula seminalis The vesicula seminalis of the rabbit is here to be understood as the
The organ consists in the
the bladder Through a
the end of a polythene
The miniature balloon
and the opposite end of
the catheter was connected to the recording instruments This technique has been used pre

TABLE I Characteristics of contractile activity in some regions of the male genital duct in rabbits Mean values ± S E

Region	Number of animals	Amplitude (mm H_2O/contraction)	Frequency (contractions min)	Duration (sec/contraction)
Ductus epididymidis	20	15 9±1 8	3 4±0 3	12 9±0 8
Vas deferens, proximal part	22	10 8±1 5	5 0±0 5	10 4±1 0
Vas deferens, distal part	17	6 2±1 2	5 7±0 6	9 5±0 9
Significance of difference between regions (Student's t test)				
Ductus epididymidis — vas deferens proximal part		$p < 0.025$	$p < 0.01$	$p < 0.05$
Ductus epididymidis — vas deferens, distal part		$p < 0.001$	$p \sim 0.001$	$p < 0.01$
Vas deferens, proximal part — vas deferens, distal part		$p < 0.025$	$p < 0.2$	$p < 0.3$

viously to register the contractions of the uterus *in vivo* (Cross 1958 and others) Only one kind of preparation was studied in each animal After finishing the surgical procedures, the abdomen [illegible] sur[illegible] du[illegible] conditions for about 20 min before the observations on the muscular activities started

Definitions and calculations

The following characteristics of contractile activity were noted (except for the vesicula seminalis)

The amplitude of the contractions, measured as the height of each contraction (variations in pressure of 1 mm H_2O or less were neglected)

The frequency, counted as the number of contractions/min

The duration, giving the time in sec from the start of each contraction to the end of its relaxation phase

The data obtained were calculated as mean values ± S E from observation periods of 3 min A p value of 0 05 or less was considered significant

Results

Ductus epididymidis and *vas deferens* Nearly all the preparations studied showed varying degrees of spontaneous, rhythmic contractile activity Only one out of 22 preparations of the proximal part of the vas deferens and one out of 17 of the distal part of the organ did not demonstrate spontaneous contractions In order to show that the contractile activity observed in the ductus epididymidis was not due to the abdominal environment of the organ, the pressure fluctuations in this region were also studied in a number of preparations, after moving the testis back into the scrotal site The procedure did not seem to alter the contractile pattern of the duct The data of the different characteristics of contractile activity of the various regions are presented in Table I

When the intraluminal pressure was kept constant, there were considerable dif-

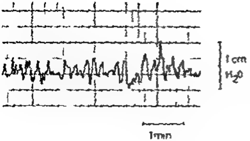

Fig 2 Contractile activity of the vesicula seminalis studied by means of an intraluminal balloon technique

ferences in the c ntractile activity between the ductus epididymidis and the proximal and the distal parts of the vas deferens (Fig 1 Table ... most regular in the d... compared with the ... vas deferens ($p < 0.05$ and $p < 0.001$ respectively) Besides there were unexpected differences in rhythmic activity between the two parts of the vas deferens Thus the amplitude was greater in the proximal part than in the distal part ($p < 0.05$) The frequency, on the other hand was significantly lower in the ductus epididymidis than in both the proximal ($p < 0.01$) and the distal ($p \sim 0.001$) parts of the vas deferens Compared with the ductus epididymidis the duration of each contraction decreased in the proximal part of the vas deferens ($p < 0.05$) as well as in the distal part of this organ ($p < 0.01$) In addition the muscular activity of the genital duct (measured as the product of amplitude and frequency) was calculated and found to differ between regions Thus the distal part of the vas deferens showed a lower degree of activity than the proximal part ($p < 0.025$) and the ductus epididymidis ($p < 0.001$)

Apparently there was a general tendency to an increase in the magnitude and duration of the rhythmic contractions as well as of the activity but a decrease in frequency towards the proximal part of the male genital duct system

Vesicula seminalis All preparations but one out of the 13 studied showed spontaneous but in most cases irregular contractions of rather low magnitude (Fig 2) The frequency and the duration were about the same as those of the distal part of the vas deferens However the different technique used in registering the contractions of the vesicula seems to make any comparison irrelevant

Discussion

The present observations show that the male accessory reproductive organs are capable of spontaneous contractions *in vivo* and indicate a gradual increase of the amplitude of the contractions as well as of muscular activity towards the testis This tendency is also manifested in the small amplitudes found in the vesicula seminalis

There may well be underlying hormonal factors to account for these phenomena Thus there have been reported inhibitory effects of androgen hormones on the con

tractile activity of the vas deferens and the vesicula seminalis (Martins and Valle 1939, Martins Valle and Porto 1940 Kniseh, Grunt and Berry 1958) but stimulating effects of these substances on the ductus epididymidis (Risley 1959) and the seminiferous tubules (Niemi and Karmano 1965)

It seems probable that at least the vas deferens is normally quiescent, unless activated by neuronal mechanisms Hence the possibility must be taken into account that the observed muscular activities, as well as the differences found between the regions studied may have been conditioned by the experimental situation For instance differences in muscular morphology between the ductus epididymidis and the vas deferens may contribute to a different contractile pattern when exposed to the same intraluminal pressure On the other hand, the observed differences in the amplitude and in the muscular activity in the proximal and distal parts of the vas deferens seem more difficult to explain on these grounds However, according to Goerttler (1934), the muscle elements of the duct in man assume a more circular character distally In spite of the probable myogenic nature of the contractions, the observed phenomena may be related to differences in the innervation of the two regions of the vas deferens (Hodson 1964, Norberg Risley and Ungerstedt 1967) as well as to variations in the biophysical properties of the different segments of the organ (Melton and Salvidar 1962) Thus it may be that the vas deferens, like the oviduct uterus complex is divided functionally into several regions which may contract independently and asynchronously

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For helpful criticism I am very much indebted to Dr J E Kihlstrom and Professor P E Lindahl

References

Cross B A The motility and reactivity of the oestrogenized rabbit uterus *in vivo* with comparative observations on milk ejection *J Endocr* 1958 *16* 237—260
Goerttler K Die Konstruktion der Wand des menschlichen Samenleiters und ihre funktionelle Bedeutung *Gegenbaurs morph Jb* 1934 *74* 550—580
Hodson N Sympathetic nerves and reproductive organs in the male rabbit *J Reprod Fertil* 1965 *10* 209—220
Knisely W H J A Grunt and E J Berry *In vivo* observations on the seminal vesicles of adult neonatally castrated rats before and after intravenous testosterone *Amer J Physiol* 1957 *189* 437—440
Martins T and J R Valle Endocrine control of the motility of the male accessory genital organs *Endocrinology* 1939 25 80—90
Martins T J R Valle and A Porto Pharmacology *in vitro* of the human vasa deferentia and epididymis the question of the endocrine control of the motility of the male accessory genitals *J Urol* (Baltimore) 1940 *44* 682—697
Melton C E and J T Salvidar Impulses in smooth muscle of rabbit deferent duct *Amer J Physiol* 1962 *203* 853—856
Niemi M and M Karmano Contractility of the seminiferous tubule of the postnatal rat testis and its response to oxytocin *Ann Med exp Fenn* 1965 *43* 40—42
Norberg K A P L Risley and U Ungerstedt Adrenergic ennervation of the male reproductive ducts in some mammals *Z Zellforsch* 1967 *76* 278—286
Risley P L Hormone effects of the *in vivo* contractile behaviour of the ductus epididymidis *Anat Rec* 1959 *133* 329—330
Risley P L Physiology of the male accessory organs In *Mechanisms concerned with Conception* Ed C G Hartman Pe

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From the Brain Research Laboratory, E blocket, the University Hospital Lund, and from the Departments of Neurosurgery, Universities of Lund and Gothenburg, Sweden

The Effect of Hypovolemic Hypotension on Extra- and Intracellular Acid-Base Parameters and Energy Metabolites in the Rat Brain

By

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Abstract

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The [illegible]
cell [illegible]
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or p [illegible]
there was a small gradual increase in tissue lactate concentration and in the lactate/pyruvate ratio, even at a moderate decrease in blood pressure The lactate and pyruvate changes were significant even after corrections for blood and CSF lactate and pyruvate contents, and similar changes were seen in the CSF The moderate changes in extra and intracellular lactate concentrations did not lead to any noticeable decreases in calculated extra and intracellular pH values since even moderate reductions in blood pressure usually led to small concomitant falls in the tissue CO_2 tension Thus if it can be assumed that the cerebral blood flow was upheld at the moderately reduced perfusion pressures the results strongly speak against the possibility that the decrease in cerebrovascular resistance which occurs during autoregulation of flow in hypovolemic hypotension is related to extracellular pH changes

It is well known that the brain is efficiently protected against variations in its energy supply by means of mechanisms which maintain its blood flow in spite of a falling blood pressure or of an increasing intracranial pressure Thus, it was reported a long time ago that the cerebral blood flow does not decrease significantly until the mean blood pressure is reduced to 60—70 mm Hg (for review, see Lassen 1959) Recent animals experiments have confirmed these earlier observations but they have shown that the cerebral circulation is not significantly decreased until an arterial hypotension, or an intracranial hypertension, has decreased the cerebral perfusion pressure to about 40 mm Hg (Haggendal and Johansson 1965, Harper 1965, Haggendal *et al* 1969)

The circulatory studies quoted have lately been corroborated by studies which have shown that there are no changes in brain tissue metabolites such as lactate, phosphocreatine, ATP, ADP, and AMP until the blood pressure falls below 40—50 mm Hg (Roth *et al* 1967 Reulen *et al* 1968) At still lower perfusion pressures there are marked metabolic changes and marked increases in the intra and extracellular acidities (Reulen *et al* 1968, Kaasik *et al* 1969 b Zwetnow 1969), and below a perfusion pressure of 25 mm Hg irreversible tissue damage has been reported to occur (Brierley *et al* 1968)

Since previous studies of the effect of arterial hypotension upon brain tissue metabolites (Roth *et al* 1967, Reulen *et al* 1968) utilized techniques which did not allow an accurate analysis of small metabolic changes at mean blood pressures exceeding 40 mm Hg we have determined the tissue concentrations of lactate pyruvate, phosphocreatine, ATP, ADP and AMP after a 20 min period of an accurately controlled mean pressure of from 160 to 30 mm Hg using fixation and extraction techniques which minimized autolytic changes The experiments also allowed extra- and intracellular acid base changes to be assessed and intracellular NADH/NAD ratios to be calculated

Methods

[illegible] 300 400 g The [illegible]

recorded on a chart recorder The equipment was calibrated against a column of water before and after each experiment Mean pressures were obtained by means of electrical integration The rectal temperature was measured with an electrothermometer and intermittant heating was applied so as to keep the temperature of the animal as close to 37° C as possible

In all experiments a steady state period of 20—30 min was allowed before the animals were slowly bled from the arterial cannula into a heparinized glass syringe When the desired mean blood pressure was obtained (usually within 3—5 min) cautious withdrawals and infusions were made so that the mean blood pressure was held as constant as possible for 15—20 min At the end of the hypotensive period CSF was sampled and the brain was frozen Arterial blood samples were taken before and during the hypotensive period and also just prior to sampling of CSF Control experiments were run by using unbled animals studied during the same period and also by prolonging the hypotensive period to 1 hr

All methods and techniques used in the present study have been described previously (see e g Kjallquist et al 1969 a Kaasik et al 1969 a and b) and the following account will there- [illegible]

measured

Cisternal cerebrospinal fluid (CSF) obtained by puncture of the exposed atlantooccipital membrane was either analysed for lactate and pyruvate for the total CO_2 content or for the CO_2 tension (see below)

In each experiment the brain tissue was frozen *in situ* with liquid nitrogen and subsequently analysed for the total CO and the water contents and for the lactate pyruvate phosphocreatine ATP ADP and AMP concentrations All these labile metabolites were measured after extraction of the tissue at −10° to −15° C (Lowry *et al* 1964), using enzymatic technique

and continuous recording of each enzymatic curve (Hohorst *et al* 1959) Since the NADH used contained AMP as a contamination (Lowry *et al* 1964), a blank sample was run for each individual AMP analysis

The intracellular bicarbonate concentration—$(HCO_3^-)_i$—, and the equivalent intracellular "pH" (pH_i'), were calculated according to the following equations

$$(HCO_3^-)_i = \frac{TCO_2 - ptCO_2 \cdot 0.029 - 0.12\,(HCO_3^-)_{csf} - 0.03\,(HCO_3^-)_{bl}}{0.64} \qquad (1)$$

$$pH_i' = 6.120 + \log \frac{(HCO_3^-)_i}{ptCO_2 \cdot 0.0314} \qquad (2)$$

In these equations TCO

CO₂ tension in mm

the CO₂ solubility

0.12 the assumed

0.64 the weight of

cellular water Since the normal relations between the CSF and the arterial CO_2 tensions (Ponten and Siesjo 1966) are not upheld in hypotension (see below, and Kaasik *et al* 1969 b) the intracellular pH was calculated only in those rats in which the CSF CO_2 tension was

(Kaasik *et al* 1969 b) communication Since the blood lactate and pyruvate concentrations were not measured in all experiments, a similar procedure was followed for the blood corrections The critical factor in this corrections was the lactate concentration, but since there was a very good linear correlation between the decrease in the blood buffer base and the increase in the lactate concentration, intracellular lactate and pyruvate concentrations could be derived for all those experiments in which measurements of the arterial pH were made (see Methods)

The intracellular (cytoplasmatic) $NADH/NAD^+$ ratio was calculated according to the formula (see Granholm and Siesjo 1969, Kaasik *et al* 1969 a)

$$\frac{NADH}{NAD^+} = \frac{(Lact)}{(Pyr)} \cdot \frac{1.11 \cdot 10^{-11}}{(H^+)_i}$$

where lactate and pyruvate denote intracellular concentrations $(H^+)_i$ the negative antilogarithm of pH_i and $1.11 \cdot 10^{-11}$ the apparent equilibrium constant as determined by Williamson *et al* (1967)

Results

In the majority of the present experiments the initial arterial pCO_2 was between 30 and 40 mm Hg and the pO_2 between 100 and 140 mm Hg In no case was the initial pCO_2 lower than 30 mm Hg or the pO_2 lower than 80 mm Hg A few animals with a rectal temperature exceeding 38° C one with an initial hemoglobin concentration of less than 10 g/100 ml and two with arterial CO_2 tensions exceeding 45 mm Hg were excluded from the material In each animal the blood pressure was continuously recorded both before and during the hypotensive period In the majority of the experiments the blood pressure could be upheld at the desired levels with very small fluctuations (± 10 mm Hg) and very little of the blood had to be reinfused If unduly large variations occurred or if a substantial part of the blood had to be reinfused to keep the blood pressure constant, the rat was discarded

The induced arterial hypotension invariably led to changes in the arterial CO_2

tension in the blood buffer base, and in the hemoglobin concentration Thus at a mean arterial blood pressure of 100 mm Hg there was a decrease in the CO tension of about 4 mm Hg and a base excess of about −8 meq/l while at a mean blood pressure of 60 mm Hg the CO tension had decreased by about 10 mm Hg and the base excess was about −14 meq/l When the mean blood pressure was decreased by 40 and 80 mm Hg the hemoglobin concentration decreased by about 1 5 and 3 g/100 ml respectively the maximal decreases obtained being about 5 g/100 ml Thus even at mean pressures of 30—50 mm Hg the arterial hemoglobin concentration usually did not fall below 10 g/100 ml

Fig 1 shows the measured tissue concentrations of phosphocreatine ATP ADP and AMP at the various mean arterial pressures studied There were no apparent changes in these metabolites until the mean pressure fell below 40 mm Hg and it should be specifically pointed out that no single phosphocreatine value lower than 4 75 and no single AMP value higher than 0 05 mmoles kg tissue were recorded above this blood pressure There are thus very abrupt changes in phosphocreatine ATP and AMP when the blood pressure falls below 40 mm Hg (the filled symbols in the figure represent the mean values reported by Kaasik *et al* 1969 b for a mean blood pressure of 25—35 mm Hg)

In Fig 2 the tissue lactate and pyruvate concentrations and the lactate/pyruvate ratios have been related to the mean arterial pressure The results indicate a small but gradual increase in the lactate concentration at decreasing blood pressures accelerating at pressures below about 60 mm Hg and a continuous increase in the lactate/pyruvate ratio Thus although there were no drastic changes in these parameters until the pressure fell below 40 mm Hg there appeared to be small but clearcut changes in the tissue lactate concentration and in the lactate pyruvate ratio at reductions in pressure which were unaccompanied by changes in phosphocreatine, ATP ADP and AMP

Since part of the changes in lactate and pyruvate may have been due to the corresponding and larger changes in the blood and CSF compartments the intracellular lactate and pyruvate concentrations were calculated as described in the methods However these corrections did not influence the tentative conclusion that a gradual reduction of the blood pressure from normal values leads to a gradual increase in the (intracellular) lactate concentration and in the lactate/pyruvate ratio (Fig 3) Thus the lines of best fit to the points indicate that when the blood pressure is reduced to 80—90 mm Hg the intracellular lactate concentration increases by about 1 mmole/kg i e water and the lactate/pyruvate ratio by 2—3 units At a mean pressure of 40—50 mm Hg the corresponding increases were about 2 mmoles/kg and about 5 units respectively

Analyses on cisternal CSF showed changes similar to those observed in the tissues *i e* gradual increases in the lactate concentration and in the lactate/pyruvate ratio with falling blood pressure (Fig 4 open circles) However there were no or very small changes in the CSF pyruvate concentration Thus although the increase in the CSF lactate was very similar to that calculated for the intracellular space the

and continuous recording of each enzymatic curve (Hohorst *et al* 1959) Since the NADH used contained AMP as a contamination (Lowry *et al* 1964), a blank sample was run for each individual AMP analysis

The intracellular bicarbonate concentration—$(HCO_3^-)_i$—, and the equivalent intracellular pH (pH_i) were calculated according to the following equations

$$(HCO_3^-)_i = \frac{TCO_2 - ptCO_2 \cdot 0.029 - 0.12\,(HCO_3^-)_{CSF} - 0.03\,(HCO_3^-)_{bl}}{0.64} \tag{1}$$

$$pH_i = 6.120 + \log \frac{(HCO_3^-)_i}{ptCO_2 \cdot 0.0314} \tag{2}$$

In these equation TCO_2 is the total CO_2 content (µmoles/g of wet tissue) $ptCO_2$ the tissue CO_2 tension in mm Hg (considered to be equal to the CSF CO_2 tension), 0.0292 and 0.0314 the CO_2 solubility in wet tissue and in intracellular water, respectively (µmoles/g/mm Hg) 0.12 the assumed extracellular volume (i e 12 %), 0.03 the assumed blood volume (i e 3 %) 0.64 the weight of the intracellular water phase and 6.120 the pK_1' of carbonic acid in intracellular water Since the normal relations between the CSF and the arterial CO_2 tensions (Ponten and Siesjo 1966) are not upheld in hypotension (see below, and Kaasik *et al* 1969 b) the intracellular pH was calculated only in those rats in which the CSF CO_2 tension was measured directly

The intracellular lactate and pyruvate concentrations were calculated in the same way as was the intracellular bicarbonate concentration using corrections for the lactate and pyruvate contained in the blood and in the CSF volumes of the tissue However, in order to allow a calculation of intracellular lactate and pyruvate concentrations for those animals in which the pH was calculated (and in which thus the CSF CO_2 tension was measured), the CSF lactate and pyruvate concentrations were derived from a curve relating the CSF lactate and pyruvate concentrations to the mean arterial blood pressure obtained in the present and in a previous (Kaasik *et al* 1969 b) communication Since the blood lactate and pyruvate concentrations were not measured in all experiments a similar procedure was followed for the blood corrections The critical factor in this corrections was the lactate concentration but since there was a very good linear correlation between the decrease in the blood buffer base and the increase in the lactate concentration intracellular lactate and pyruvate concentrations could be derived for all those experiments in which measurements of the arterial pH were made (see Methods)

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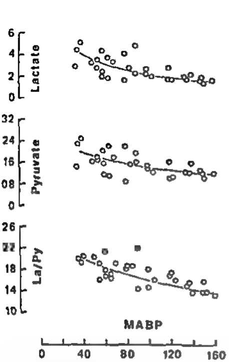


Fig 3

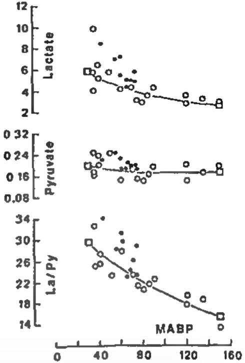


Fig 4

Fig 3 The relation between the mean arterial blood pressure and the intracellular lactate and pyruvate concentrations and lactate/pyruvate ratios (see Fig 1 and 2) The values were calculated by correcting for the amounts of lactate and pyruvate contained in the blood and CSF volumes of the tissue (see Methods) The corrections did not influence the conclusions drawn from the experiments illustrated in Fig 2

Fig 4 The CSF lactate and pyruvate concentrations related to the mean arterial blood pressure during a hypovolemic hypotension of 20 (unfilled circles or 60 (filled circles) min duration (*cf* Fig 1, 2 and 3) The squares denote mean concentrations reported by Kaasik *et al* (1969 b) The increase in the CSF lactate concentration at decreasing blood pressure was similar to that calculated for the intracellular space but the CSF lactate/pyruvate ratio increased even more steeply than did the intracellular ratio

showed that the difference between the CSF and the arterial CO_2 tensions increased gradually with falling mean arterial blood pressure (Fig 5) For example at a mean arterial pressure of 40—50 mm Hg the CSF CO_2 tension was 10—15 mm Hg higher than the arterial CO_2 tension

The total CO_2 content of the cisternal CSF was measured in 12 expts (Table I) The experiments were divided into 3 groups of 4 animals each with mean arterial pressures of 85, 65 and 50 mm Hg, respectively When the results were analysed it was found that the two groups with the highest blood pressures had bicarbonate concentrations and calculated pH values which were not significantly different from those of normal rats under nitrous oxide anesthesia (27 meq/kg and 7 45, see Kjällquist *et al* 1969 a) Only in the group with the lowest blood pressure did the bicarbonate concentration indicate a moderate acidosis

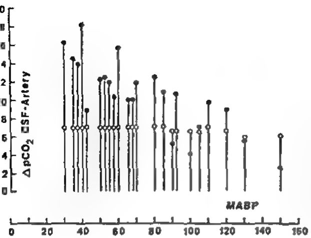


Fig. 5. The CSF arterial pCO_2 difference related to the mean arterial blood pressure during a hypovolemic hypotension of 20 min duration. The pCO_2 difference increased gradually with falling blood pressure (filled circles). The "normal" pCO_2 differences, i.e. those expected from the prevailing arterial CO_2 tensions (see Pontén and Siesjö 1966), are also given in the figure (open circles).

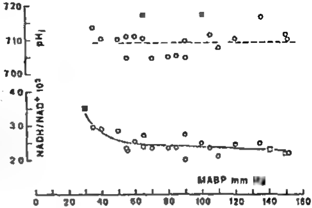
The intracellular "pH" was calculated in 22 expts. The results showed that the pH_i' either remained normal (about 7.09, see Kjällquist *et al.* 1969 a) or even increased slightly at mean blood pressures down to 40 mm Hg. Thus, the acidifying effect of an increased lactate concentration (see above) seemed to be fully compensated by a decrease in the CO_2 tension (Fig. 6).

Since intracellular "pH" values, and intracellular lactate and pyruvate concentrations were derived for the same experiments the intracellular NADH/NAD ratios could be calculated. The ratios calculated have been related to the mean arterial blood pressure in the lower part of Fig. 6. The results suggest either no increases or very small increases in the $NADH/NAD^+$ ratio with falling blood pressure (*cf.* increases obtained at mean pressures of 25—35 mm Hg, as shown by filled symbol in Fig. 6).

TABLE I. Acid base changes in cisternal CSF of rats during hypovolemic hypotension (M ± S.E.M.). The animals were divided into 3 groups of 4 animals each, covering the blood pressure range of 95 to 45 mm Hg. The CSF pH was calculated from the total CO_2 content and from the CSF CO_2 tension, which was either measured on the same animal (4 expts.) or derived from Fig. 5.

Art. blood		Cisternal CSF			
MABP mm Hg	P_{CO_2} mm Hg	T_{CO_2} mm Hg	P_{CO_2} mm Hg	HCO_3^- mEq/kg	pH
75—95 (4)	32 ±0.8	28.2 ±0.6	41.9 ±0.4	26.9 ±0.6	7.44 ±0.03
60—70 (4)	31 ±1.2	28.0 ±0.2	40.8 ±2.0	26.8 ±0.2	7.45 ±0.03
45—55 (4)	28 ±1.5	26.4 ±0.2	39.8 ±2.1	25.2 ±0.3	7.43 ±0.03

Fig ■ The calculated intracellular pH (pH), and the calculated intracellular (cytoplasmatic) NADH/NAD ratio related to the mean arterial blood pressure in hypovolemic hypotension The interrupted line denotes normal intracellular pH , as obtained by Kaasik *et al* (1969 a) The squares denote calculated NADH/NAD ratios from Kaasik *et al* (1969 b)



Discussion

The present experiments have shown that if the mean arterial blood pressure of artificially ventilated rats is decreased for a period of about 20 min, there are no significant changes in the tissue concentrations of phosphocreatine, ATP, ADP and AMP until the mean pressure falls below 35—40 mm Hg These results are therefore in complete agreement with those of Ruelen *et al* (1968) Thus although these authors reported phosphocreatine and AMP concentrations which must have been influenced by autolytic changes during the fixation of the tissue (*cf* also Roth *et al* 1967), their conclusions regarding the tissue phosphates appear completely valid Taken together, our results seem to establish that an arterial hypotension which does not extend below 40 mm Hg is unaccompanied by any gross alteration in the oxygenation of the brain The results are then also in accordance with those showing that the cerebral circulation is very little or not all decreased in this pressure range (Haggendal and Johansson 1965 Harper 1965 Haggendal *et al* 1969)

Although the present results show that there are no changes in the tissue phosphates above a mean arterial pressure of about 40 mm Hg they strongly indicate that there is a continuous increase in the tissue lactate concentration and in the lactate/pyruvate ratio when the blood pressure is decreased from the normal range of 120—160 mm Hg This conclusion is supported both by the derived intracellular changes, and by the concomitant extracellular changes Evidently these changes may be at least partly due to the decrease in the hemoglobin concentration and possibly also in the CO tension However it is less probable that the lactate/pyruvate changes in the extra and intracellular compartments could be due to a passive exchange with the blood especially since these changes can be observed already 5—10 min after the commencement of the hypotension (unpublished observations) It should also be recalled that the present CSF measurements fully confirm the increases in the CSF lactate/pyruvate ratio observed after small decreases in the cerebral perfusion pressure in dogs (Siesjo *et al* 1968 Kjallquist *et al* 1969 b) In these experiments the lactate/

pyruvate changes were also seen when the perfusion pressure was decreased by means of increasing the intracranial pressure, a condition which is unaccompanied by changes in the blood lactate/pyruvate ratio

If we accept the existence of definite changes in the intracellular lactate concentration and in the intracellular lactate/pyruvate ratio even at very moderate decreases in the mean arterial blood pressure we are faced with the apparent paradox that the same blood pressure level is unaccompanied by significant changes in the phosphocreatine or AMP concentrations Thus both the phosphocreatine concentration (Hohorst *et al* 1968) and the AMP concentration (Minard and Davis 1962) have been claimed to be sensitive indicators of the presence of tissue hypoxia

The present results do not allow the above discrepancy to be explained The calculation of intracellular lactate pyruvate and bicarbonate concentrations and of NADH/NAD ratios is based on assumptions regarding the size of the extracellular fluid volume and regarding the homogeneity of the intracellular fluids the validity of which is unknown It should also be kept in mind that all the parameters measured including the concentrations of the labile phosphates, pertain to the brain as a whole and it is quite possible that more profound metabolic changes occur in certain regions of the brain especially those situated between the distribution fields of major arteries (*cf* Brierley *et al* 1969) Until such regions have been explored we must leave the interpretation of the lactate/pyruvate changes remembering also that the NADH/NAD ratios calculated are probably not sufficiently accurate to disclose very small changes in the oxido reduction state of the cells Thus, an increase in the lactate/pyruvate ratio from 14 to 16 5 at constant pH (*e g* 7 09) would correspond to a NADH/NAD increase from 2 06 to 2 32 a change which cannot be resolved ess very large materials are employed

The moderate increases in the extra and intracellular lactate concentrations were insufficient to give any significant changes in the calculated extra and intracellular pH at mean blood pressures exceeding about 50 mm Hg but the pH constancy was probably partly due to the slight lowering of the CSF CO_2 tension caused by the hypotension The constancy of the CSF pH has an interesting bearing on the mechanisms responsible for the maintenance of the cerebral blood flow at reduced arterial pressures (autoregulation) Thus it has been suggested that the extracellular H concentration is the main regulator of cerebrovascular resistance (see discussion in Symposium on CSF and CBF 1968 ed Ingvar *et al*) We can tentatively assess the relation between cerebrovascular resistance and extracellular pH by recalling that 5—7 % CO_2 in the inspired air approximately doubles blood flow Thus if this degree of hypercapnia increases the CSF CO_2 tension by about 15 mm Hg during spontaneous ventilation the CSF pH must fall by about 0 15 units when the cerebrovascular resistance is reduced to half If the extracellular H regulates the cerebral blood flow and if the flow is upheld at a mean blood pressure of 60 mm Hg the decrease in the cerebrovascular resistance would again be expected to be associated with a fall in pH of about 0 15 units The fact that no or very small CSF pH changes were observed evidently speaks against a H mechanism

in the maintenance of cerebral blood flow during hypotension (see discussion in Ingvar *et al* 1968)

Although the present results strongly suggest that changes in the extracellular pH do not explain the cerebral vasodilation during arterial hypotension they do not allow any conclusions regarding the constancy of flow Thus the gradual increase in the difference between the CSF and the arterial CO_2 tensions at decreasing blood pressures could be due to a decreased carbon dioxide transport capacity of the blood concomitant to the decrease in the hemoglobin concentration but part of it might also be due to a small decrease in flow If such a decrease exists even at moderate reductions of the cerebral perfusion pressure it cannot though be responsible for any larger functional or metabolic consequences

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References

BRIERLEY J B A W BROWN BARBARA J EXCELL and B S MELDRUM Brain damage in the [illegible]

GI [illegible]

HAGGENDAL E and B JOHANSSON Effects of arterial carbon dioxide tension and oxygen saturation on cerebral blood flow autoregulation in dogs *Acta physiol scand* 1965 *66* Suppl 258 27—53

HAGGENDAL E J LOFGREN N J NILSSON and N N ZWETNOW Effects of varied cerebrospinal fluid pressure on cerebral blood flow in dogs *Acta physiol scand* 1970 *79* In press

HARPER A M Physiology of cerebral blood flow *Brit J Anaesth* 1965 *37* 225—235

HOHORST H J F H KREUTZ and T BUCHER Über Metabolitgehalte und Metabolit Konzentrationen in der Leber der Ratte *Biochem Z* 1959 *332* 18—46

INGVAR D H N A LASSEN B K SIESJO and S SKINHOJ eds International Symposium on CBF and CSF *Scand J clin Lab Invest* 1968 Suppl 102 VIII A—K

KAASIK A L NILSSON and B K SIESJO The effects of asphyxia upon the lactate pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970 a *78* 433—447

KAASIK A L NILSSON and B K SIESJO The effect of arterial hypotension upon the lactate pyruvate and bicarbonate concentrations of the brain tissue and cisternal CSF and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970 b *78* 448—458

KJALLQUIST A, M NARDINI and B K SIESJO The regulation of extra and intracellular acid base parameters in the rat brain during hyper and hypocapnia *Acta physiol scand* 1969 a *76* 485—494

KJALLQUIST A B K SIESJO and N N ZWETNOW Effects of increased intracranial pressure on cerebral blood flow and on cerebrospinal fluid HCO_3 pH lactate and pyruvate in dogs *Acta physiol scand* 1969 b *75* 345—352

LASSEN N A Cerebral blood flow and oxygen consumption in man *Physiol Rev* 1959 *39* 183—238

LOWRY O H I V PASSONEAU F X HASSELBERGER and D W SCHULTZ Effect of ischemia on known substrates and cofactors of the glucolytic pathway in brain *J biol Chem* 1964 *239* 18—30

MINARD F N and R V DAVIS The effects of electroshock on the acid soluble phosphates of rat brain *J biol Chem* 1962 *237* 1283—1289

PONTEN U and B K SIESJO Gradients of CO_2 tension in the brain *Acta physiol scand* 1966 *67* 129—140

Reulen, H. J., U. Steude, W. Brendel and F. Medzihradsky. Elektrolyt und Metabolitkonzentrationen im Gehirn nach normovolamischer Drucksenkung. *Z. ges. exp. Med.* 1968. *146*. 241—260.
Roth, E., H. W. Schuler, O. Suleder and B. Sobol. Metabolitkonzentrationen in Herz, Gehirn, Niere und Leber des Hundes bei kontrollierter Hypotension. *Z. ges. exp. Med.* 1967. *144*. 258—272.
Siesjo, B. K., A. Kjallquist and N. N. Zwetnow, The CSF lactate/pyruvate ratio in cerebral hypoxia. *Life scie.* 1968. *7*. 45—52.
Williamson, D. H., Patricia Lund and H. A. Krebs. The redox state of free nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* 1967. *103*. 514—527.
Zwetnow, N. N. The influence of an increased intracranial pressure on the lactate, pyruvate, bicarbonate, phosphocreatine, ATP, ADP and AMP concentrations of the cerebral cortex of dogs. *Acta physiol. scand.* 1970. *79*. In press.

Acta physiol scand 1970 79 125—128

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Isolation of ^{32}P-Labelled Phosphorylserine from Ehrlich Mouse-Ascites Tumour Cells Suspended in an Isotonic Medium Containing ^{32}P-Labelled Adenosine Triphosphate

By

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Abstract

Ågren G and G Ronquist *Isolation of ^{32}P labeled phosphorylserine from Ehrlich mouse ascites tumour cells suspended in an isotonic medium containing ^{32}P labelled adenosine triphosphate* Acta physiol scand 1970 *79* 125—128

[illegible]

in the tumour cell membrane

It has recently been shown that human erythrocytes yeast cells and tumour cells are capable of forming extracellular ATP when incubated in an isotonic medium containing all the necessary substrates and cofactors of the first energy yielding step of glycolysis (Ronquist 1968 Ågren and Ronquist 1968 Ågren and Ronquist 1969) Short incubation times were used These results supported the view that the newly formed extracellular ATP was generated by the enzymes concerned located at the surface of the cells

Extracellular ATP can also be split into ADP and orthophosphate by intact tumour ascites cells (Ronquist and Ågren 1969) thus indicating the presence of an ATPase enzyme system located at the cell surface *(cf* Clarkson and Maizels 1952 Herbert 1956 Hoffman 1962)

Brief exposure of NaATPase preparations to ^{32}P ATP in the presence of Na and Mg^{++} results in the incorporation of the ^{32}P phosphoryl group into the acid precipitable material (Albers *et al* 1963 Post *et al* 1964) Furthermore Ahmed and Judah (1965) isolated ^{32}P labelled phosphorylserine from a hydrolysate of a brain lipoprotein incubated with γ ^{32}P ATP They had reason to believe that a cation stimulated ATPase in the preparation had formed an intermediate phosphoryl enzyme where the linkage to protein was stable to acid hydrolysis

The aim of the present investigation was to find out whether the terminal phosphoryl group of extracellular ATP could be incorporated into a phosphorylserine group of any membrane protein of intact Ehrlich mouse ascites tumour cells suspended in an isotonic medium.

Material and methods

The Ehrlich mouse ascites tumour cells were obtained and prepared as previously described (Ågren and Ronquist 1969) except that the third washing was performed with a solution containing 10 % Dextran T 40 (M_w = 41 800, M_n = 25 700, $[\eta]$ = 0.210, AB Pharmacia, Uppsala, Sweden) instead of bovine serum albumin. The tumour cell suspension to be incubated also contained this solution in the same proportions as given previously (Ågren and Ronquist 1969). The incubation experiments followed immediately after the preparatory procedure.

The tumour cells were incubated anaerobically for various times in the same way as previously described (Ågren and Ronquist 1969). However, in a series of experiments the tumour cells were also incubated with 0.4 μmoles of γ-^{32}P-ATP (specific activity about 5 mCi/μmole) together with the *incomplete system* (Ågren and Ronquist 1969). On these occasions the orthophosphate of the incomplete system was unlabelled.

All incubations were terminated by adding 3.0 ml of 3 N perchloric acid to the cell suspensions, followed by 10 ml of ice-cold distilled water. The acid soluble material was separated from the acid insoluble material by centrifugation and decantation (Ågren and Ronquist 1969). γ-^{32}P-ATP was prepared according to Engstrom (1962). ^{32}P-phosphorylserine was isolated by chromatography on Dowex 50 after hydrolysis of the Schneider protein fraction from the acid insoluble material as previously described (Ågren *et al.* 1962).

Radioactivity was measured in accordance with an earlier work (Ronquist 1967).

Results

Table I shows that maximum ^{32}P-labelled phosphorylserine was obtained with 30 seconds incubation of the tumour cell suspension with the incomplete system containing ^{32}P-ATP. These data give evidence for an intermediate binding of the terminal phosphoryl group of ATP to phosphorylserine of a membrane protein of the tumour cells. Labelled ^{32}P-phosphorylserine was also formed when the tumour cells were incubated with the complete system, in which extracellular ^{32}P-ATP can be formed (Ågren and Ronquist 1969). The differences in the amounts of labelled ^{32}P-phosphorylserine may be explained by the differences in composition between the incomplete system containing γ-^{32}P-ATP and the complete system.

In the cases where the tumour cells were incubated with the incomplete system only, in which extracellular ^{32}P-ATP cannot be formed (Ågren and Ronquist 1969), no measurable labelled ^{32}P-phosphorylserine could be isolated after short incubation periods. However, with 5 min incubation with the incomplete system containing labelled orthophosphate small amounts of labelled phosphorylserine were isolated. It is probable that the orthophosphate during this incubation time had penetrated the cell membrane (*cf.* Ågren and Ronquist 1969) and thereafter had been incorporated into an intracellular phosphoprotein. This finding should be compared with the work of Kennedy and Smith (1954), who incubated washed intact Ehrlich ascites tumour cells in an isotonic medium containing ^{32}P-orthophosphate for 2 hrs at 37° C. They found with this relatively long incubation period that ^{32}P-phosphorylserine with high specific activity could be isolated from a cellular phosphoprotein fraction.

For comparison, experiments were performed with human erythrocytes which also

TABLE I Incorporation of the terminal phosphoryl group of extracellular γ-^{32}P-ATP into a phosphorylserine residue by intact Ehrlich mouse ascites tumour cells

	5 sec SerP	30 sec SerP	60 sec SerP	5 min SerP
γ-^{32}P-ATP + incomplete system	0.82	2.57	1.43	1.[illegible]
complete system	0.19	1.13	1.03	1.18
incomplete system	<0.01	<0.01	0.01	0.03

Ehrlich mouse ascites tumour cells incubated in three different isotonic mediums (see text) for various times at 37 °C. The mediums contained 1×10^{-3} M ^{32}P-orthophosphate. In the medium to which γ-^{32}P-ATP was added the orthophosphate was unlabelled. The incubations were terminated with perchloric acid and labelled ^{32}P-phosphorylserine (SerP) was isolated from the acid insoluble material as given in Methods. The figures (mean values of three experiments) denote the phosphoryl incorporation into phosphorylserine in μmoles $\times 10^{-6}$ per mg dry weight (Schneider protein). For further details, see text.

are capable of forming extracellular ATP although to a much lesser extent (Ronquist 1968). The intact erythrocytes were therefore incubated under similar conditions as the tumour cells. No measurable amounts of labelled ^{32}P-phosphorylserine could however be isolated (*i.e.* $<0.01 \times 10^{-6}$ μmoles of labelled phosphorylserine per mg dry weight (Schneider protein)).

Table II illustrates the close relationship between the temperature of the tumour cells incubated with the complete system and the amounts of labelled ^{32}P-phosphorylserine formed.

Discussion

The results of the present investigation clearly show that the labelled phosphoryl group of γ-^{32}P-ATP can be linked to a serine residue of tumour cells incubated under isotonic conditions with ^{32}P-ATP in the extracellular medium. Since ATP cannot penetrate the cell membrane (see *e.g.* Ronquist 1968, Agren and Ronquist 1969) it is reasonable to infer that the terminal phosphoryl group had reacted with a surface located membrane protein. It is also clear from the figures of the tables that ortho-

TABLE II Effect of temperature on the labelling of ^{32}P-phosphorylserine from tumour cells incubated with complete and incomplete system

	37 °C SerP	20 °C SerP	4 °C SerP
complete system	1.13	0.21	0.02
incomplete system	0.01	<0.01*	<0.01*

Ehrlich tumour ascites cells incubated with complete and incomplete system (see text) for 30 seconds at different temperatures. The figures (mean values of three experiments, unless otherwise indicated) denote the phosphoryl incorporation into phosphorylserine (SerP) in μmoles $\times 10^{-6}$ per mg dry weight (Schneider protein). For further details, see text of table I.

* single experiments

phosphate cannot react in a similar manner unless having first been linked into a high energy bound as exists in ATP Therefore, labelled ^{32}P-phosphorylserine could be isolated from tumour cells incubated with the complete system but not from those incubated with the incomplete system The short incubation times of thirty seconds with maximum incorporation of phosphoryl groups into phosphorylserine give evidence that the reaction between extracellular ATP and the protein at or in the tumour cell membrane is of enzymatic nature This concept is also supported by the results given in Table II Extracellular ATP can thus act as the donor of a high energy phosphoryl group to a serine residue of a membrane protein This energy source could be available for different energy dependent reactions of the cell membrane as *e g* conformational changes (Kavanau 1965), contractions or movements of the membrane (Benedetti and Emmelot 1968) or active transport (Albers 1967)

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References

ÅGREN G B HALLBERG and G RONQUIST The incorporation of phosphorus into human erythrocyte ghosts *Acta chem scand* 1962 *16* 1770—1780

ÅGREN G and G RONQUIST, Formation of extracellular adenosine triphosphate by yeast cells *Acta physiol scand* 1968 *74* 394—397

ÅGREN G and G RONQUIST Formation of extracellular adenosine triphosphate by tumour cells *Acta physiol scand* 1969 *75* 124—128

AHMED K and J D JUDAH Identification of active phosphoprotein in a cation activated adenosine triphosphatase *Biochim biophys Acta* (Amst) 1965 *104* 112—120

ALBERS R W Biochemical aspects of active transport *Ann Rev Biochem* 1967 *36* 727—756

ALBERS R W S FAHN and G J KOVAL The role of sodium ions in the activation of electrophorus electric organ adenosine triphosphatase *Proc nat Acad Sci* (Wash) 1963 *50* 474—481

BENEDETTI E L and P EMMELOT *Structure and function of plasma membranes isolated from liver* In The membranes Ultrastructure in biological systems 1968 Vol 4 pp 33—120 Ed by A J Dalton and F Haguenau Academic Press New York

CLARKSON E M and M MAIZELS Distribution of phosphatases in human erythrocytes *J Physiol* Lond 1952 *116* 112—128

ENGSTRÖM L Formation of a phosphorylenzyme during hydrolysis of ^{32}P labelled glucose 6 phosphate by alkaline phosphatase from calf intestinal mucosa and *Escherichia coli* *Arkiv Kemi* 1962 *19* 129—140

HERBERT E A study of the liberation of orthophosphate from ATP by stromata of human erythrocytes *J cell comp Physiol* 1956 *47* 28—60

HOFFMAN J F Cation transport and structure of the red cell plasma membrane *Circulation* 1962 *26* 1201—1210

KAVANAU J L *On gross aspects of membrane transformations* In Structure and function in biological membranes 1965 Vol 1 pp 66—73 Holden Day Inc

KAVANAU J L *Transformation between the open and closed configurations* In Structure and function in biological membranes 1965 Vol 2 pp 326—353 Holden Day Inc

KENNEDY E P and W SMITH The isolation of radioactive phosphoserine from "phosphoprotein" of the Ehrlich ascites tumor *J biol Chem* 1954 *207* 153—163

POST R L A K SEN and A S ROSENTHAL A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes *J biol Chem* 1965 *240* 143 — 1445

RONQUIST, G Formation of adenosine triphosphate by a membrane fraction from human erythrocytes *Acta chem scand* 1967 *21* 1484—1494

RONQUIST G Formation of extracellular adenosine triphosphate by human erythrocytes *Acta physiol scand* 1968 *74* 594—605

RONQUIST, G and G ÅGREN Unpublished 1969

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Salivary Glands of the Rat in a Hot Environment

By

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Abstract

Elmér, M and P Ohlin *Salivary glands of the rat in a hot environment* Acta physiol scand 1970 *79* 129—132

A marked hypertrophy of the normally innervated submaxillary gland was observed in rats subjected to heat (34° C) for 3 weeks The increase in weight of parasympathetically denervated glands was much less while that of sympathetically denervated glands was still pronounced The size of both sympathetically and parasympathetically denervated glands was not affected in a hot environment It seems likely that the hypertrophy of the submaxillary gland is [illegible] a large extent secondary to an increased secretory activity elicited by the parasympathetic secretory fibres A corresponding increase in weight of the sublingual gland was not seen

Rats in a hot environment have been shown to spread saliva on their skin thereby counteracting a rise in body temperature In desalivated animals the heat resistance is markedly lowered and at the same time the evaporative water loss is reduced (Hainsworth 1967 and 1968 Hainsworth *et al* 1968) These findings indicate an increased secretory activity of the salivary glands in the heat This ought to give a glandular hypertrophy according to the hypothesis that the size of salivary glands is determined by the organ activity (see Elmer and Ohlin 1969) In the present experiments rats were subjected to heat for 3 weeks and the weights of the submaxillary and sublingual glands were determined A marked hypertrophy of the submaxillary gland was noticed in preliminary experiments To evaluate the role of the autonomic glandular nerv[illegible] in this hyperthrophy the glands on one side were parasympathetically or sympathetically denervated or both

Methods

103 rats of [illegible] Spr[illegible] Da[illegible] and a [illegible]olony bred at th[illegible] In[illegible] and [illegible] sexes were used T[illegible]

When the [illegible] were denervated [illegible] para sympathetic [illegible] post ganglionic sympathetic denervation by extirpation of the superior cervical ganglion [illegible] rats served as controls while 5? were placed in a room with a constant temperature of 34° C f[illegible]

TABLE I The Weight of Normally Innervated, Parasympathetically and Sympathetically Denervated Submaxillary Glands in Rats Subjected to a Hot Environment for 3 Weeks and in Controls. The Weight of the Glands is Expressed as Dry Weight per 100 g Body Weight. Values are M±S.E.M., and number of glands

			Gland Weight (mg)			
			Hot Environment		Control	
Sex	Strain	Denervation	Denervated	Innervated	Denervated	Innervated
Female	Institute	Parasympathetic	16 ±1.3[1] (11)	31.0±0.83[3] (11)	12.2±0.43 (9)	19.5±0.50 (9)
Male	"	"	13.6±0.37[1] (8)	24.2±0.56[3] (8)	12.0±0.41 (5)	17.6±0.[illegible] (5)
Male	Sprague Dawley		13.7±0.91 (6)	24 ±2.1[1] (6)	11.7±0.99 (9)	16.5±0.78 (11)
Female	Institute	Sympathetic	24.8±0.86[3] (12)	25.9±0.81[3] (13)	19.7±0.56 (9)	19.3±0.60 (10)
Male	Sprague-Dawley		16.0±0.65[2] (6)	21.2±0.96[3] (6)	13.1±0.54 (7)	[illegible]7±0.67 (7)
Male	"	Parasympathetic + Sympathetic	10.3±0.60 (8)	22 ±1.6[3] (8)	11.3±0.33 (9)	17.1±0.73 (9)

[1] <0.05 — [2] 0.01 — [3] <0.001 when compared with corresponding Control.

3 weeks. When aged 101 days all the rats were killed by a blow on the neck. The submaxillary and sublingual glands were carefully dissected and weighed (wet weight). The glands were heated to 110° C for 48 hrs to get the dry weight which gives a more reliable value than the wet weight (Ohlin 1965). The weight of the glands was expressed as dry weight per 100 g b.w. A few glands had to be disregarded due to subcapsular bleedings caused by the blow. The body weight was determined at the start and at the end of all experiments.

Results

Submaxillary gland. In the controls parasympathetic denervation was found to cause a denervation atrophy of about 33 % in accordance with previous findings (see Ohlin 1965). Rats which had been subjected to the hot environment for 3 weeks showed a marked increase in the weight of the normally innervated glands which was enlarged by almost 50 %. On the other hand the weight of the denervated glands was less markedly increased, unsignificantly in one group out of three (Table I).

Sympathetic denervation caused no significant change of the weight in one group of the controls but a significant (P<0.001) decrease in weight in the other group (Table I). Sympathetic denervation has previously been found to give a slight

TABLE II The Weight of Normally Innervated, Parasympathetically and Sympathetically Denervated Sublingual Glands in Rats Subjected to a Hot Environment for 3 Weeks and in Controls The Weight of the Glands is Expressed as Dry Weight per 100 g Body Weight Values are M±S E M , and number of glands

			Gland Weight (mg)			
			Hot Environment		Control	
Sex	Strain	Denervation	Denervated	Innervated	Denervated	Innervated
Female	Institute	Para sympathetic	2 5±0 20[1] (10)	4 2±0 07 (10)	2 4±0 33[1] (9)	4 8±0 19 (9)
Male	,,	,	1 7±0 09[1] (8)	3 0±0 13 (8)	1 9±0 14[1] (5)	3 3±0 13 (4)
Female	,	Sympathetic	4 4±0 20 (13)	4 2±0 17 (13)	4 4±0 18 (10)	4 1±0 15 (10)
Male	Sprague Dawley	,	2 8±0 10 (5)	3 4±0 27 (5)	3 0±0 25 (6)	3 3±0 19 (6)
Male	,	Para sympathetic + Sympathetic	2 0±0 18[1] (8)	3 2±0 09 (8)	1 6±0 05[1] (9)	3 5±0 15 (9)

[1] <0 001 when compared with the corresponding innervated glands

atrophy (Ohlin 1965 and 1968) A pronounced increase in weight, about 25 %, of both innervated and denervated glands was observed in the hot environment (Table I)

Both parasympathetic and sympathetic denervation caused an atrophy of about 34 % in controls In the hot environment the completely denervated glands showed no significant change in weight while the normally innervated contralateral glands were markedly enlarged (Table I)

Sublingual gland Section of the chorda lingual nerve caused an atrophy by about 47 % while extirpation of the superior cervical ganglion did not affect the weight of the denervated glands in controls The hot environment for 3 weeks caused no change in the size of either innervated or denervated glands (Table II)

Body weight During the experimental period the controls gained weight while the animals subjected to the hot environment lost The difference in weight between rats in the heat and controls was about 12 % for the strain bred at this Institute and about twice as much for the Sprague Dawley rats When aged 101 days the controls weighed 165 (females bred at this Institute) 242 (males bred at this Institute) and 278 g (males Sprague Dawley) as a mean The corresponding figures for the rats in the hot environment were 143 214 and 200 g respectively

Discussion

The submaxillary gland of rats increased in weight when the animals were subjected to the hot environment for 3 weeks. The hypertrophy is most likely due to an increased secretory activity in the heat for two reasons. Firstly, the experiments by Hainsworth *et al.* (1968) indicate an increased salivary secretion in a hot environment. Secondly, the size of salivary glands seems to be dependent on the organ activity (see Elmér and Ohlin 1969).

Reflex secretion in rats may be elicited not only via the parasympathetic but also via the sympathetic glandular nerves (Ohlin 1968). Therefore, it seemed of interest to find out whether the hypertrophy of the submaxillary gland in the hot environment was affected by parasympathetic or sympathetic denervation. The sympathetically denervated gland increased markedly in weight in the hot environment while the parasympathetically denervated gland was much less affected. Thus, it seems likely to conclude that the hypertrophy of the submaxillary gland is mainly caused by an increased secretory activity induced by the parasympathetic secretory fibres. It seems likely, however, that also reflex secretion via the sympathetic glandular nerves is of some importance since in the hot environment the sympathetically innervated but parasympathetically denervated gland was slightly increased in size while the completely denervated gland was not. Further, it should be pointed out that the weight of the normally innervated gland is increased twice as much after parasympathetic than after sympathetic denervation. This may be due to the pronounced reduction of the secretory capacity of the parasympathetically denervated gland which has to be compensated by the contralateral gland while after sympathetic denervation the denervated gland still retains a marked secretory capacity.

The sublingual gland decreased markedly in weight after parasympathetic denervation while it was unaffected by sympathetic denervation. It is interesting to note that the glandular elements of the sublingual gland have no sympathetic innervation in rats (Norberg and Olson 1965).

The sublingual gland, contrary to the submaxillary gland, showed no hypertrophy in the hot environment. The weights of corresponding control and experimental glands were similar when they were expressed per 100 g b.w.

References

ELMÉR, M. and P. OHLIN, Compensatory hypertrophy of the rat's submaxillary gland. *Acta physiol. scand.* 1969. *76*. 396—398.

HAINSWORTH, F. R., Saliva spreading, activity, and body temperature regulation in the rat. *Amer. J. Physiol.* 196[illegible]. *21*[illegible]. 1288—1292.

HAINSWORTH, F. R., Evaporative water loss from rats in the heat. *Amer. J. Physiol.* 1968. *214*. 979—982.

HAINSWORTH, F. R., E. M. STRICKER and A. N. EPSTEIN, Water metabolism of rats in the heat: dehydration and drinking. *Amer. J. Physiol.* 1968. *214*. 983—989.

NORBERG, K. A. and L. OLSON, Adrenergic innervation of the salivary glands in the rat. *Z. Zellforsch.* 1965. *68*. 183—18[illegible].

OHLIN, P., Secretory responses of innervated and denervated submaxillary glands of rats. *Acta univ. Lund.* II. 1965. *2*[illegible]. 1—12.

OHLIN, P., Sympathetic secretory innervation of the rat's submaxillary gland. *Quart. J. exp. Physiol.* 1968. *53*. 19—22.

Acta physiol. scand. 1970. 79. 133—135

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Effects on Water Diuresis of Infusions of Transmitter Substances into the 3rd Ventricle

By

KERSTIN OLSSON

It has been shown that the supraoptic and paraventricular nuclei, among a few other hypothalamic structures, contain very high densities of catecholamines, mainly of noradrenaline (*cf.* Fuxe and Hökfelt 1969). Since these nuclei are involved in the production and the secretion of antidiuretic hormone it seemed of interest to study if infusions of catecholamines into the 3rd brain ventricle would inhibit the water diuresis of hydrated goats.

It was also of interest to study if intraventricular infusions of other transmitter substances, especially of acetylcholine, would inhibit the water diuresis. Acetylcholine has earlier been shown to release antidiuretic hormone when injected intracarotidly (Abrahams and Pickford 1956) or into the supraoptic nuclei (Pickford 1947).

Methods

Three adult female goats (b.w. about 35 kg) were used in 19 expts. The goats were confined in metabolism cages having free access to hay and water. They were all prepared with permanent cannulae for infusions into the anterior part of the 3rd brain ventricle. The operation and infusion techniques were described earlier (Andersson *et al.* 1967). The rate of infusion was maintained at 10 μl/min. In all experiments cerebrospinal fluid was observed to drain out of the cannula on compression of the neck both before and after the infusion. Thus a completely free mixing of the infused solution with the fluid of the 3rd ventricle was guaranteed.

Solutions of the following substances were infused: l-noradrenaline bitartrate, l-adrenaline bitartrate, 3-hydroxytyramine HCl (dopamine), acetylcholine chloride, 5-hydroxytryptamine creatinine sulfate (serotonin) and sodium bitartrate. All substances were made up in 0.6 % NaCl except for serotonin which had to be solved in a small amount of 0.01 N NaOH before it was added to the NaCl solution.

A water diuresis was established by giving the goats 100 ml of tepid water/kg b.w. by stomach tube 60 to 90 min before urine collection was started. The urine was collected via a retention catheter inserted into the urinary bladder. Urine osmolality was determined by the use of a Knauer osmometer.

Results

Noradrenaline and adrenaline. Noradrenaline was infused at rates of 0.6 and 0.7 μg/kg b.w./min. The infusion periods lasted for 3 or 10 min. The results of a 10 min infusion (0.7 μg/kg b.w./min) is shown in Fig. 1 (above, right). This infusion produced a marked inhibition of the water diuresis and a conspicuous rise in urine osmolality

Acta physiol. scand. 1970. 79. 136—138

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Regulation of Intracellular pH in the Rat Brain in Chronic Hypercapnia

By

K. MESSETER AND B. K. SIESJÖ

It is known that the buffer capacity of brain intracellular fluids in acute hyper- and hypocapnia defined as d log pCO_2/d pH, is much too high to be accounted for by physicochemical buffering alone (Roos 1965, Kjallquist *et al* 1969, Granholm and Ponten 1969), and both previous and recent experiments have shown that there is an appreciable increase in the bicarbonate concentration of whole brain tissue in chronic hypercapnia (Nicholls 1958, Weyne *et al* 1968) These results suggest that mechanisms exist which give rise to a regulation of intracellular pH, but they do not allow any conclusions regarding the efficiency of the pH regulation in chronic hypercapnia or regarding its mechanisms In order to analyse the intracellular acid-base regula n we have determined bicarbonate concentrations and calculated pH values for cerebral extra and intracellular spaces in sustained hypercapnia.

Rats weighing 250—350 g, were exposed to hypercapnia for 3 and 24 hrs in a Perspex box which was perfused with gas mixtures containing 30 % O_2 and about 5 and 10 % CO respectively About 30 min before the end of the intended exposure period the rats were taken from the box anesthetized with divinyl ether, tracheotomized immobilized with tubocurarine chloride, and connected to an animal respirator Immediately after the tracheotomy a gas mixture was administered, containing 30 % O 60—65 % N_2O and either 5 or 10 % CO_2 During this procedure the ventilation was set so as to give an arterial CO_2 tension close to that obtained while the rats were exposed to carbon dioxide in the box At the end of the exposure period cisternal *cerebrospinal fluid* was sampled, and the brain was frozen *in situ* with liquid nitrogen The total CO_2 content was measured in CSF and in the supratentorial parts of the brain and the bicarbonate concentration as well as the pH in the extra- and intracellular spaces were calculated using a tissue CO_2 tension derived from the measured arterial CO_2 tension, and [illegible] 12 % extracellular volume (for details, see Kjallquist [illegible]

The figure illustrates th [illegible] cell [illegible] concentrations, and the pH values, in 8 groups [illegible] to [illegible] for 3 and 24 hrs respectively and compares [illegible] measured in normocapnic control animals [illegible] % CO_2 for

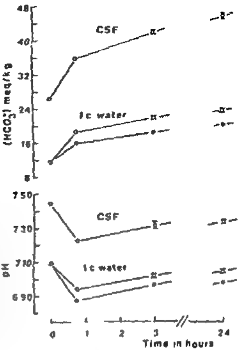

FIG 1 The calculated bicarbonate concentrations, and the calculated pH values, for the extra and intracellular spaces of the brain of rats exposed to about 10 % CO_2 for 0 75, 3 and 24 hrs, respectively Note gradual return of pH_i in spite of the continuous hypercapnia (tissue CO_2 tension about 90 mm Hg), and the simultaneous increases of the extra and intracellular bicarbonate concentrations The filled circles represent intracellular parameters calculated on the assumption of a passive exchange of bicarbonate at a constant electrochemical potential difference for H^+ ions

45 min (see Kjallquist *et al* 1969) The figure shows that both the CSF and the intracellular bicarbonate concentrations increased with time, and that the intracellular pH' almost had returned to the control value at 24 hrs in spite of the continuous hypercapnia

In order to analyse the mechanisms responsible for the regulation of pH_i the electrochemical (H) difference between the extra and intracellular spaces ($\Delta\mu H$) was calculated according to the equation

$$\Delta\mu H = 61\ 5\ (pH_{i}\ _{l}-pH\ ) + \eta$$

where η is the transmembrane potential If we assume a transmembrane potential of -70 mV, and if we take the CSF pH and the pH to be 7 45 and 7 10 respectively at a CO_2 tension of 40 mm Hg (Kjallquist *et al* 1969) $\Delta\mu_{H^+}$ is calculated to $-48\ 5$ mV If we further assume that η and $\Delta\mu_{H^+}$ remain constant in chronic hypercapnia we may now calculate the changes in the intracellular bicarbonate concentration and in pH_i which would be expected on the basis of a passive exchange of H or HCO_3 between the extra- and intracellular compartments These expected changes have been entered as closed circles in the figure The analysis shows that the gradual increase in the intracellular bicarbonate concentration was parallel to that expected on the basis of a passive exchange, and thus suggests that the gradual increase in the CSF bicarbonate concentration is of great importance for the regulation of pH However at any given occasion the pH_{i} and the intracellular bicarbonate concentratio

were higher than would be expected if $\Delta\mu_{H^+}$ or φ remained constant This suggests that hypercapnia either gives rise to a generalized depolarisation of the transmembrane potentials or to an increase in $\Delta\mu_{H^+}$ In other words, hypercapnia may well increase an active transport of hydrogen ions from the cells to the extracellular fluids

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References

GRANHOLM L and U PONTEN The *in vivo* CO_2 buffer curve of the intracellular space of cat cerebral cortex *Acta neurol scand* 1969 *45* 493—501

KJALLQUIST A M NARDINI and B K SIESJO The regulation of extra and intracellular acid base parameters in the rat brain during hyper and hypocapnia *Acta physiol scand* 1969 *76* 485—494

NICHOLLS G Serial changes in tissue carbon dioxide content during acute respiratory acidosis *J clin Invest* 1958 *37* 1111—1122

ROOS A Intracellular pH and intracellular buffering power of the cat brain *Amer J Physiol* 1965 *209* 1233—1246

WEYNE J G DEMEESTER and I LEUSEN Bicarbonate and chloride shifts in rat brain during acute and prolonged respiratory acid base changes *Arch int Physiol Biochim* 1968 *76* 415—433

Acta physiol. scand. 1970 79. 139—141

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Inhibition by Prostaglandin E_2 of Sympathetic Neurotransmission in the Rabbit Heart

By

P HEDQVIST, L STJÄRNE and Å WENNMALM

Prostaglandin E_2 (PGE_2) is a powerful vasodilator in many species (cf Bergström Carlson and Weeks 1968) I v injection causes a fall in blood pressure and an increase in heart rate and in cardiac output (Bergstrom and Euler 1963 Weeks, Sekhar and DuCharme 1969) In the isolated perfused rat heart PGE_2 has been reported to increase the coronary flow considerably while the effects on heart rate and contractile force were small and inconsistent (Vergroesen deBoer and Gottenbos 1967) Recent reports indicate that PGE_2 may modulate the function of sympathetically innervated tissues Thus PGE_2 reduces in a dose-dependent fashion the release of noradrenaline (NA) from the cat spleen on nerve stimulation (Hedqvist 1969 1970) and inhibits the neuromuscular transmission in the guinea pig vas deferens (Euler and Hedqvist 1969)

In the present paper we wish to report that PGE_2 in moderate doses inhibits the sympathetic neurotransmission in the perfused rabbit heart

Rabbits weighing 1 4 to 2 1 kg were killed by a blow on the head, and the hearts with intact sympathetic nerve supply were dissected out Hukovic and Muscholl 1962, Löffelholz and Muscholl 1969) and perfused by the Langendorff technique with Tyrode solution of the following [illegible]

[illegible]

immediately acidified and chilled After purification on alumina aliquots were analyzed fluorimetrically for NA

Infusion of PGE_2 into the isolated perfused rabbit heart in concentrations ranging from 7×10^{-8} to $5.5 \times$ [illegible] M did not appreciably alter the flow rate through the organ. The heart rate remained unchanged in 9 expts increased moderately in 4 and decreased slightly in 1 expt The contractile force was unaffected in 10 expts while it tended to decrease in 3 and to increase in 1 expt. The outflow of NA from the spontaneously beating heart was always very low and often no NA could be detected PGE_2 did not affect this outflow Stimulation of the sympathetic nerves for periods of 30 sec at frequencies of 1 5 and 10 sec caused a frequency dependent increase in heart rate and in contractile force This effect was excellently reproducible for

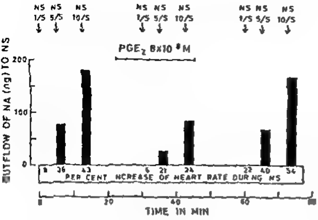

Fig 1 Perfused rabbit heart Outflow of NA and per cent increase of heart rate in response to 30 sec periods of nerve stimulation (NS) at 1 5 and 10/sec Effect of PGE_2 8×10^{-8} M

many stimulation periods During the 5 and 10/sec stimulations considerable amounts of NA appeared in the effluent from the heart Infusion of PGE_2 (8×10^{-8}—$5\,5\times10^{-7}$ M) markedly reduced the outflow of NA and distinctly counteracted the increase in heart rate to nerve stimulation (Fig 1), while the increase in myocardial contractile force was less affected After the infusion of PGE_2 the heart rate and NA overflow responses to nerve stimulation increased again towards the preinfusion level (Fig 1) In 9 expts the outflow of NA to 10/sec nerve stimulation before during and after the infusion of PGE_2 in above mentioned concentrations was 112 ± 26 38 ± 9 and 74 ± 18 ng respectively (M ± S E M) The corresponding increase in heart rate was 93 ± 8 55 ± 6 and 81 ± 7 beats/min respectively (M ± S E M)

Atropine (10^{-6} g/ml) which was used in the majority of the experiments did not materially change the inhibitory effect of PGE_2

PGE ($9\,7\times10^{-8}$ to 6×10^{-7} M) was found to cause an inconsistent and slight reduction of the chronotropic and inotropic responses to NA (0 5 μg)

Comment

The observed effects of PGE_2 on sympathetic neutransmission and NA outflow in the rabbit heart are in agreement with previous observations in other sympathetically innervated tissues (Hedqvist 1969 Euler and Hedqvist 1969) and make it increasingly likely that PGE can inhibit neutrotransmission in sympathetically innervated tissues in general The fact that the outflow of NA in response to nerve stimulation was markedly reduced by PGE while the inhibitory action of PGE on the chronotropic and inotropic response to exogenous NA was small or absent supports the previously presented concept that the main target for PGE_2 is the process of NA release from the nerve terminals (Hedqvist 1970)

Vergroesen *et al* 1967 found a small or no direct effect of PGE_2 on rate and contractile force in the rat heart We have confirmed this observation in the rabbit heart

In view of the annulment by atropine of the inhibitory action of PGE_1 on neuromuscular transmission in the guinea pig vas deferens (Euler and Hedqvist 1969) it is interesting that atropine did not alter the effect of PGE_2 in the rabbit heart This indicates that the main target of action of PGE_1 and PGE_2 on the sympathetic neuroeffector system may differ for the two agents as previously pointed out (Hedqvist 1970) It may also be due to species differences or to differences of sympathetic innervation in the two tissues (*cf* Sjostrand 1965)

PGE_2 which is released by sympathetic nerve stimulation to the spleen (Davies Horton and Withrington 1967) appears to be present in autonomic nerves and in sympathetically innervated tissues including the heart of at least some species (Karim Hillier and Devlin 1968) The observed effects of exogenous PGE_2 in the present study are thus compatible with the concept that endogenous PGE_2 acts as modulator of sympathetic nerve transmission (Hedqvist 1969 1970

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References

BERGSTROM S, L A CARLSSON and J R WEEKS The prostaglandins A family of biologically active lipids *Pharmacol Rev* 1968 *20* 1—48

BERGSTROM S and U S v EULER The biological activity of prostaglandin E_1 E_2 and E_3 *Acta physiol scand* 1963 *59* 493—494

DAVIES B N E W HORTON and P G WITHRINGTON The occurrence of prostaglandin E_2 in splenic venous blood of the dog following splenic nerve stimulation *J Physiol* (Lond) 1967 *188* 38—39P

EULER U S v and P HEDQVIST Inhibitory action of prostaglandin E_1 and E_2 on the neuromuscular transmission in the guinea pig vas deferens *Acta physiol scand* 1969 *77* 510—512

HEDQVIST P Modulating effect of prostaglandin E_2 on noradrenaline release from the isolated cat spleen. *Acta physiol scand* 1969 *75* 511—512

HEDQVIST P, Control by prostaglandin E of sympathetic neurotransmission in the spleen *Life Sci* 1970 *9* 269—278

HUKOVIC S and E MUSCHOLL Die Noradrenalin Abgabe aus dem isolierten Kaninchen herzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung *Naunyn Schmiedeberg's Arch exp Path Pharmak* 1962 *244* 81—96

KARIM S M M K HILLIER and J DEVLIN Distribution of prostaglandins E_1 E_2 $F_{1\alpha}$ and $F_{2\alpha}$ in some animal tissues *J Pharm Pharmacol* 1968 *20* 749—753

LOFFELHOLTZ K and E MUSCHOLL A muscarinic inhibition of the noradrenaline release evoked by postganglionic sympathetic nerve stimulation *Naunyn-Schmiedeberg's Arch Pharmak* 1969 *265* 1—15

SJOSTRAND N O The adrenergic innervation of the vas deferens and the accessory male genital glands *Acta physiol scand* 1965 *65* Suppl 257 1—82

VERGROESEN A J J DE BOER and J J GOTTENBOS Effects of prostaglandins on perfused isolated rat hearts In *Prostaglandins* Proc 2nd Nobel Symp Stockholm June 1966 *Ed* S Bergstrom and B Samuelsson pp 211—218 Almqvist and Wiksell Stockholm Interscience New York 196

WEEKS J R N C SEKHAR and D W DUCHARME Relative activity of prostaglandins E_1 E_2 and A_2 on lipolysis platelet aggregation smooth muscle and the cardiovascular system *J Pharm Pharmacol* 1969 *21* 103 108

Acta physiol. scand. 1970. 79. 142—144

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Permeability of Vascular Smooth Muscle Cells to Non-Electrolytes

By

BORJE JOHANSSON

The smooth muscle cells in the portal vein of the rat adjust their intracellular fluid volume in response to moderate changes of extracellular tonicity in the way one would expect from "perfect osmometers", and these variations in the volume of the *cells are accompanied by characteristic alterations in their spontaneous electrical and* mechanical activity (Johansson and Jonsson 1968, Arvill, Johansson and Jonsson 1969, Jonsson 1969 a, b) Swelling of the cells in hypotonic solutions is associated with depolarization and a sustained increase in muscle activity whereas hyper-osmotic shrinkage due to non-permeant substances, such as sucrose, leads to hyper polarization and inhibition Anisosmolar solutions containing urea were found to use transient shifts in muscle activity which were attributed to the short lasting *ffects on cell volume that this permeant molecule would exert*

The rate of penetration of urea ^{14}C into the smooth muscle cells was measured by Arvill *et al* (1969) and their data indicate a permeability constant for urea of about 3×10^{-7} cm/sec, assuming a volume to surface ratio of 10^{-4} cm in these cells The transient changes in muscle activity produced by anisosmolar urea Krebs showed, in their time-course, a good correlation to the rate of penetration of urea as measured by urea ^{14}C

It seemed therefore that a comparative analysis of the smooth muscle responses to anisosmolar solutions of various non electrolytes would offer a simple indirect way of estimating the relative rates of penetration of such molecules into the cells This approach was used in a recent study of a limited number of organic substances and the following order of permeability was obtained sucrose < erythritol < glycerol < urea < thiourea < ethylene glycol (Johansson 1969 a) The present series of experiments was carried out as an extension of the former study Information has now been obtained for a greater number of test substances and their relative rates of penetration have been quantified approximately on the basis of the duration of the transient changes of muscle activity

The mechanical responses of isolated rat portal veins to anisosmolar Krebs solutions, containing different non electrolytes were recorded as in the previous set of experiments (Johansson 1969 a . To allow comparison of the transient responses all experiments included urea as a reference substance From the time-course of the changes in muscle activity seen on administration of the anisosmolar solution and on

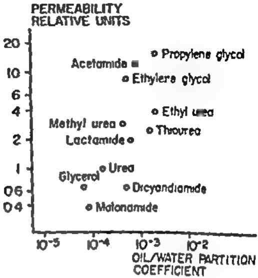

Fig 1 Permeability of vascular smooth muscle to non-electrolytes The rate of penetration of the different substances is expressed relative to that of urea which is arbitrarily set to unity The oil/water partition coefficient for the various molecules is given on the abscissa.

return to control medium approximate figures were obtained for the rates of penetration of the different molecules relative to that of urea Results are summarized in Fig 1 where relative permeabilities have been plotted *versus* the oil/water partition coefficient for the various molecules Responses to substances with still lower rates of penetration for instance erythritol seemed too slow to permit quantitative comparison with urea Conversely substances like ethylene glycol and acetamide evidently penetrated so quickly that they failed to produce any obvious effects on muscle activity under the ordinary experimental conditions They did produce osmotic transients however, if muscle excitability was enhanced (Johansson 1969 b) or if the experiments were done at a reduced temperature (+30° C) Their rates of penetration relative to that of urea have thus been obtained under such modified experimental conditions Molecules with still higher oil/water partition coefficients were not included because their influence on muscle activity could not be attributed to mere osmotic effects but indicated some kind of narcotic action (Johansson 1969 a)

Fig 1 shows a clear tendency to greater rates of penetration for substances with higher oil/water partition coefficients but also considerable differences in permeability between substances having similar partition coefficients Such differences may reflect the influence of specific chemical groups or of the molecular size There is experimental evidence that molecular size is one of the factors determining the permeability of various cell membranes to non electrolytes (for ref see Diamond and Wright 1969) and this has led to the concepts of a molecular sieve effect or of a porous membrane structure In the smooth muscle of the portal vein a faster rate of penetration for the smaller molecule is indicated for instance with regard to erythritol and sucrose which have the same oil/water partition coefficient (3×

and with regard to glycerol and malonamide which also have similar partition coefficients (7×10 and 8×10^{5} respectively) This may indicate that a molecular sieve effect controls the rate of penetration of substances with very low "lipid solubility" The polar pathways ("the pores") become less important for substances with higher oil/water partition coefficent and this latter factor seems to dominate the relative permeabilities in Fig 1 In rat erythrocytes, the molecular size as a major determinant of membrane permeability extends to the group of substances listed in Fig 1, suggesting a relatively greater importance of the polar routes in this type of cell (see Johansson 1969 b)

The present study of portal vein responses to anisosmolar solutions containing different organic substances has thus revealed certain characteristics of membrane permeability in the vascular smooth muscle The relative differences in permeability constants indicated by Fig 1 allow comparisons with similar data from other cell systems Attempts were made in the previous study (Johansson 1969 a) also to characterize the membrane permeability of the vascular smooth muscle in terms of osmotic reflexion coefficients (σ) With the indirect method used approximate values for σ were obtained only for urea and glycerol The data were used in calculations of equivalent pore radius and total pore area When compared with corresponding data from studies of red blood cells the results indicated a much smaller density of pores *in the vascular smooth muscle cells Such comparisons may be of interest* but the characterization of smooth muscle permeability in terms of the porous membrane model may otherwise be of limited value in view of the fact that "lipid solubility" appears to dominate the penetrability even of relatively hydrophilic molecules as suggested by Fig 1

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References

ARVILL A B JOHANSSON and O JONSSON Effects of hyperosmolarity on the volume of vascular smooth muscle cells and the relation between cell volume and muscle activity *Acta physiol scand* 1969 *75* 484—495

DIAMOND J M and E M WRIGHT Biological membranes the physical basis of ion and non electrolyte selectivity *Ann Rev Physiol* 1969 *31* 581—646

JOHANSSON B Permeability characteristics of vascular smooth muscle cells as revealed by their osmotic responses to non-electrolytes *Acta physiol scand* 1969 *77* 282—297

JOHANSSON B *Osmotic properties of vascular smooth muscle cells* In Symposium on the physiology and the pharmacology of vascular neuroeffector systems Interlaken 1969 S Karger Basel/New York In press

JOHANSSON B and O JONSSON Cell volume as a factor influencing electrical and mechanical
physiol scand 1968 *72* 456—468
olated vascular smooth muscle in response to reduced
7 191—200
of isolated vascular smooth muscle in response to
JONSSON, O
reduced osmolarity *Acta physiol scand* 1969 *77* 201—211

Acta physiol scand. 1970. 79. 145—152

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Some Characteristics of Lipolysis in Dog Adipose Tissue. Effects of Noradrenaline, Prostaglandin E_1 and Nicotinic Acid

By

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Abstract

CARLSON, L A and H MICHELI *Some characteristics of lipolysis in dog adipose tissue Effects of noradrenaline, prostaglandin E_1 and nicotinic acid* Acta physiol scand 1970 79 145—152

noradrenaline

Nicotinic acid inhibited the basal lipolysis in five of ten dogs and the stimulation induced by noradrenaline in three of ten In those dogs where nicotinic acid inhibited lipolysis PGE_1 was in general also inhibitory

We have seen in recent studies that there are great differences between various species for the effects on lipolysis in adipose tissue of noradrenaline, theophylline nicotinic acid and prostaglandin E_1 (PGE_1) (Carlson 1963, Carlson *et al* 1964 Micheli 1969 Boberg *et al* 1969 Micheli *et al* 1969) The question of species differences with regard to the metabolism of adipose tissue has recently been reviewed by Rudman and *Di Girolamo (1967)* Such differences are of great biological interest from many points of view Although the dog is often used in studies on free fatty acid (FFA) mobilization dog adipose tissue has been relatively little studied in vitro In addition to this fact we had in preliminary studies observed that PGE_1 which is usually a potent inhibitor of lipolysis in vitro stimulated lipolysis in adipose tissue from some diabetic dogs as also seen in diabetic rats (Carlson and Micheli 1969) This study was done mainly to characterize the in vitro response of dog adipose tissue to two inhibitors of lipolysis PGE_1 and nicotinic acid

Material and methods

Twelve adult mongrel dogs of both sexes and fasted for 24 hrs were used in this study. At one p.m. they were anesthetized with pentobarbital i.v. (Nembutal®, Abbott), 30 mg per kg. After 20 min the abdomen was opened and the adipose tissue from the major omentum excised and washed free from blood with saline. The tissue was put on filter paper moistened with saline and cut into fragments of 50—100 mg. The pieces were transferred into incubation flasks of glass, four into each. The flasks contained 4 ml of Krebs Ringer bicarbonate buffer, pH 7.4 with 2 % albumin (Kabi, Stockholm, Sweden) and 0.1 % glucose. There was a preincubation time of around 45 min at the room temperature ad then additions were made and the flasks incubated at 37° C for one hr. The general procedures were as described before (Micheli 1969). The following substances were used: Noradrenaline (Nor-exadrin Conc®, Astra Sodertalje, Sweden), nicotinic acid (ACO, Stockholm, Sweden), the β adrenergic blocker Ko 592 (Boehringer, Mannheim, Germany). PGE_1 was kindly supplied by Prof. S. Bergstrom (Karolinska Institutet, Stockholm, Sweden). A stock solution of PGE_1, 1 mg per ml, was made in ethanol 99 % and stored at −17° C up to four months. The same amount of ethanol was added to all experiments and controls. The final ethanol concentration of the medium was 1 per cent. Glycerol release was determined enzymatically as described before (Micheli 1969). Statistical calculations were performed according to Snedecor (1956).

Results

The individual results from all experiments in the 12 dogs are given in Table I and the statistical evaluation of the results in Table II.

Effect of PGE_1 on basal lipolysis

The lipolytic response to the addition of PGE_1 varied qualitatively with the dose and differed widely from dog to dog. PGE_1 sometimes caused the expected inhibition of lipolysis, sometimes there was no significant effect and unexpectedly there was in several instances an enhancement of the glycerol release. With 0.1 μg of PGE_1 the basal lipolytic rate was significantly reduced in 4 of 10 dogs. When the dose of PGE_1 was increased to 1 μg we observed inhibition in one dog while lipolysis was stimulated in 6 dogs. With the highest dose, 10 μg of PGE_1, stimulation was obtained in 2 further dogs, i.e. in 8 of 12 dogs this high dose of PGE_1 increased the output of glycerol from adipose tissue. In several of these dogs the increase obtained with PGE_1 was of similar magnitude as that obtained with 0.1 μg of noradrenaline.

Among the 12 dogs there were two in which no significant effect was obtained with PGE_1 on basal lipolysis. In one of these (no. 3) the dose response seemed to be biphasic and the difference between the values obtained with 0.1 and 10 μg were statistically significant ($P<0.05$).

Effect of PGE_1 on noradrenaline stimulated lipolysis

Noradrenaline stimulated lipolysis in all dogs. In seven experiments where both 0.1 and 1 μg of noradrenaline were added per ml of incubation medium the smaller dose gave a submaximal stimulation of the glycerol release (Table II). On the average 0.1 μg of noradrenaline caused a release which was 48 per cent of that induced by 1 μg.

In 10 dogs both 0.1 μg of noradrenaline and 1 μg of PGE_1 were added to the incubation medium. The results are summarised in Fig. 1. When the addition of only

TABLE I The effect of prostaglandin E_1 (PGE_1) nicotinic acid (NIC), the β adrenergic blocker Ko 592 and noradrenaline (NOR) on glycerol release (μmoles/g wet wt/hr) in omental adipose tissue Mean values ± S E M The number of flasks per experiment was 6 except in dogs no 3 and no 5 where 8 and 5 were used Concentrations of the added substances are given in μg/ml of incubation medium except for Ko 592 where it was 4×10^{-4} Molar

		A	B	C	D	E	F	G	H	J	K	L	M
Additions		0	PGE_1 0.1	PGE_1 1	PGE_1 10	Ko 592	PGE_1 10 Ko 592	NOR 0.1 Ko 592	NOR 0.1	NOR 0.1 PGE_1 1	NIC 1	NOR 0.1 NIC 1	NOR 1
Dog no	1	0.63	0.19	0.20	0.27	0.75	0.75	0.78	1.15	0.34	0.36	0.58	2.58
		± .04	± .03	± .03	± .03	± .04	± .02	± .04	± .08	± .04	± .08	± .05	± .09
	2	0.50	0.32	0.39	0.48	0.37	0.46	0.51	1.29	0.50	0.38	0.84	—
		± .07	± .03	± .05	± .07	± .06	± .02	± .08	± [illegible]	± .03	± .03	± .04	
	3	0.33	0.29	0.32	0.46	0.49	0.52	0.54	2.08	0.75	0.23	0.98	3.72
		± .04	± .05	± .08	± .05	± .03	± .07	± .06	± .18	± .14	± .01	± .27	± .16
	4	0.28	0.17	0.15	0.17	0.20	0.20	0.27	0.93	0.35	0.14	1.20	—
		± .06	± .02	± .07	± .02	± .02	± .03	± .03	.08	± .04	± .03	± .05	
	5	0.87	0.67	0.84	1.59	0.77	1.27	0.86	3.10	2.53	0.57	2.90	3.67
		± .06	± .03	± .09	± .08	± .07	± .06	± .07	± .12	± .07	± .06	± .11	± .15
	6	0.83	0.65	1.17	2.05	0.81	1.48	0.63	1.53	1.70	0.54	1.76	3.11
		± .06	± .02	± .06	± .05	± .04	± .08	± .05	± .20	± .09	± .04	± .12	± .18
	7	0.22	—	0.78	0.43	—	—	—	—	—	—	—	—
		± .04		± .03	± .03								
	8	0.62	—	1.76	2.66	—	—	—	—	—	—	—	—
		± .06		± .09	± .10								
	9	0.30	0.40	0.82	1.24	—	—	—	1.21	1.27	0.28	1.23	2.25
		± .03	± .03	± .04	± .07				.06	± .10	± .01	± .08	± .14
	10	1.23	1.35	1.62	2.31	1.07	1.88	0.96	1.90	2.01	1.08	1.76	2.87
		± .06	± .08	± .13	± .13	± .06	± [illegible]	.06	± .08	± .08	± .03	± .09	± .12
	11	0.71	0.88	1.65	2.08	0.67	1.82	0.63	2.17	2.25	0.55	2.18	3.30
		± .04	± .09	± .04	± .09	± .03	± .12	.06	± .11	± .06	± .07	± .11	± .11
	12	0.22	0.21	0.35	0.76	0.28	0.58	0.35	2.43	2.38	0.20	2.44	—
		± .02	± .02	± .01	± .06	± .05	± .03	± .03	± .07	± .06	± .01	± .09	

1 μg of PGE_1 either inhibited or caused no change in basal glycerol release this amount was also a strong inhibitor of the noradrenaline induced stimulation of lipolysis (Fig 1 left part) On the other hand in all animals where 1 μg of PGE_1 stimulated basal lipolysis the noradrenaline enhanced glycerol release was not changed by PGE_1 (Fig 1 right part) This difference in response could not be related to sex or weight of animals

TABLE II P values for the statistical significance of the difference between the experiments from Table I. Letters indicate wich groups in Table I that are compared. (+) indicates increase and (—) decrease. H/M = ratio of delta values from basal

Compared exper	B—A	C—A	D—A	E—A	F—D	G—H	J—H	K—A	L—H	H/M in %
Dog no 1	(—) <0.001	(—) <0.001	(—) <0.001	ns	ns	(—) <0.01	(—) <0.001	(—) <0.05	(—) <0.001	27
2	(—) <0.001	ns	ns	ns	ns	(—) <0.001	(—) <0.001	(—) <0.05	(—) <0.001	—
3	ns	ns	ns	(+) 0.05	ns	(—) <0.001	(—) <0.001	ns	(—) <0.01	52
4	ns	ns	ns	ns	ns	(—) <0.001	(—) <0.001	ns	(+) <0.05	—
5	(—) <0.05	ns	(+) <0.001	ns	(—) <0.05	(—) <0.001	(—) <0.01	(—) <0.01	ns	80
6	(—) <0.05	(+) <0.01	(+) <0.001	ns	(—) <0.001	(—) <0.05	ns	(—) <0.01	ns	31
7	—	ns	(+) <0.01	—	—	—	—	—	—	—
8	—	(+) 0.001	(+) <0.001	—	—	—	—	—	—	—
9	ns	(+) 0.001	(+) <0.001	—	—	—	ns	ns	ns	47
10	ns	(+) 0.05	(+) <0.001	ns	(—) <0.05	(—) <0.001	ns	(—) <0.05	[illegible]	41
11	ns	(+) <0.001	(+) <0.001	ns	ns	(—) <0.001	ns	ns	ns	56
12	ns	(+) <0.001	(+) <0.001	ns	ns	(—) <0.001	ns	ns	ns	—

Effect of Kö 592 on basal and stimulated lipolysis

The effect of the β adrenergic blocker Ko 592 was tested alone and together with 10 μg per ml of PGE_1 and 0.1 μg per ml of noradrenaline. On the basal state Kö 592 had no effect in 8 of 9 animals. In one dog it was slightly stimulatory. In 4 or 5 animals the stimulation of lipolysis elicited by 10 ug per ml of PGE_1 was slightly but significantly inhibited by Ko 592. But the glycerol release stimulated by noradrenaline was always completely suppressed by Ko 592.

Effect of nicotinic acid on basal lipolysis

Nicotinic acid was only tested with a concentration of 1 μg per ml incubation medium. Nicotinic acid had a significantly inhibitory effect on the basal release of glycerol in 5 of 10 dogs and no effect in the other five. It might be of interest to note

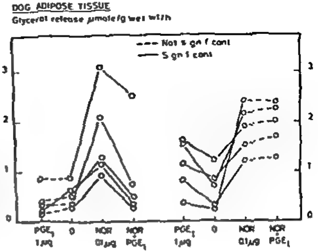

Fig 1 The effect of PGE_1 on basal and on noradrenaline (NOR) stimulated glycerol release (µmoles g wet wt hr) in omental adipose tissue from ten fasted dogs *Left part* five dogs where PGE_1 either inhibited or was without effect on basal lipolysis *Right part* five other dogs where PGE_1 stimulated basal lipolysis

that the mean glycerol release was only 0 37 in the latter 5 dogs compared to 0 81 µmole per g per hr in the first five in which nicotinic acid had an inhibitory effect It is also of interest that four of the five animals where nicotinic acid was inhibitory were the only animals where the lowest concentration of PGE_1 also significantly lowered the basal glycerol output

Effect of nicotinic acid on noradrenaline stimulated lipolysis

In those 10 dogs where the effect of PGE_1 on noradrenaline stimulated lipolysis was tested we also studied the effect of nicotinic acid in this regard In 3 of these 10 dogs nicotinic acid inhibited the noradrenaline enhancement of glycerol release In all these three PGE_1 was also inhibitory

In adipose tissue from 6 dogs nicotinic acid did not change the noradrenaline stimulated lipolysis It is noteworthy that in all these 6 dogs 10 µg of PGE_1 increased the glycerol release It is also of interest to look at the effect of 1 µg of PGE_1 in the noradrenaline stimulated lipolysis in these six dogs As a matter of fact in 5 of them also PGE_1 was completely ineffective against noradrenaline and in one PGE_1 had a very slight effect In addition it should be mentioned that in 2 of these 6 dogs nicotinic acid had caused a significant lowering of basal glycerol release

In the remaining dog nicotinic acid had a slight increasing effect above that caused by only noradrenaline If this unusual response was due to technical reasons or not is not known

Discussion

Effects of nicotinic acid

Noradrenaline increased lipolysis in all dogs which is in accord with our experiences in vivo with several hundred dogs over the last five years Carlson and Orö 1962 Carlson, Liljedahl and Wirsen 1965 Carlson *et al* 1969) Nicotinic acid decreased

basal lipolysis in five of ten dogs and inhibited noradrenaline stimulated lipolysis only in three of ten dogs which is in contrast with our experience in vivo where we since 1961 (Carlson and Oro 1962, Carlson *et al* 1969) in more than hundred dog experiments always have had a complete inhibition of noradrenaline stimulated FFA mobilization with nicotinic acid In these in vivo studies noradrenaline has been given as a constant infusion at a dose giving maximal increases in FFA and glycerol levels in plasma Nicotinic acid has been effective when given intravenously in doses of 2 mg/kg or more In the present in vitro studies we know that in five of the dogs where nicotinic acid was ineffective on the noradrenaline stimulated lipolysis, this lipolytic rate was not maximal since a higher dose of noradrenaline caused a further increase in glycerol release It should also be mentioned that in our experience with rat adipose tissue in vitro with similar incubation conditions 0 02—0 04 μg nicotinic acid per ml usually gives a 50 per cent inhibition of basal and of noradrenaline stimulated lipolysis and 0 1 μg gives maximal inhibition (Carlson, unpublished)

These findings raise the question if nicotinic acid inhibits FFA mobilization by a direct action in adipose tissue as suggested before (Carlson 1963, Carlson 1965) or if the action is indirect The following findings were in favour of a direct action 1) A rapid uptake of nicotinic acid by adipose tissue after i v (Carlson and Hanngren 1964) and S C (Carlson and Nye 1966) administration 2) The consistent inhibitory action of nicotinic acid on basal as well as noradrenaline stimulated lipolysis obtained in vitro with rat adipose tissue (Carlson 1963 Carlson, unpublished data) 3) The prevention by nicotinic acid of the usual increase of cyclic-AMP levels caused by catecholamines in rat adipose tissue in vitro (Butcher *et al* 1968) In dogs, however, point 2 is not fulfilled although nicotinic acid in vivo consistently inhibits the catecholamine stimulated lipolysis The present results necessitate further studies on the mode of action of nicotinic acid The lack of effect of nicotinic acid on noradrenaline stimulated lipolysis is however probably not due to a low rate of penetration into the tissue during in vitro conditions since in two dogs nicotinic acid was effective on basal but not on noradrenaline stimulated lipolysis

Effects of PGE$_1$

The actions of PGE$_1$ on lipolysis are more complex than those of nicotinic acid both in vivo and vitro In vivo in fasted dogs PGE$_1$ has a dual effect When infused at low doses the plasma levels of FFA and glycerol increased while infusion of higher doses decreased these plasma levels (Bergstrom *et al* 1966 b) Although the present in vitro study also indicated a dual effect of PGE$_1$ in at least some of the dogs this was in the opposite direction The lowest dose of PGE$_1$ 0 1 μg per ml, inhibited lipolysis in four dogs while the highest stimulated in 8 dogs The dual effect seen in dogs in vivo has been explained as follows (Carlson 1967) At low doses PGE$_1$ stimulated the activity of the sympathetic nervous system which enhanced lipolysis in adipose tissue At the higher doses however PGE$_1$ accumulated in adipose tissue and eventually the antilipolytic effect of PGE$_1$ abolished the stimulation The possibility that the stimulation of lipolysis in vitro caused already with 1 μg of PGE$_1$ could be due

to actions on the sympathetic nervous system with release of noradrenaline was tested with addition of the β adrenergic blocking agent Ko 592 (Stock and Westermann 1966) This agent had only a slight inhibitory action on the increased glycerol output obtained with PGE_1 but caused a complete suppression of that with noradrenaline This suggests that, contrary to in vivo, the increased lipolysis after PGE_1 in vitro was not related to catecholamines It is possible that the slight inhibition of PGE_1 stimulated lipolysis was due to the high concentration of Ko 592 This high concentration might inhibit lipolysis by interfering with the action of cyclic-AMP on the lipase, like α adrenergic blocking agents and not only by inhibiting the stimulation of formation of cyclic AMP induced by catecholamines (Aulich *et al* 1967)

It could certainly be argued that the effects with 10 μg of PGE_1 are unphysiological In general tissue levels of the prostaglandins are at the most around 1 μg per g although seminal plasma contains 100 μg/ml or more (*cf* Bergstrom *et al* 1968) However as nothing is known about the penetration of PGE_1 into adipose tissue cells the effects with 0 1 μg of PGE_1 might be associated with membrane effects while those seen with the higher doses might be due to intracellular effects Furthermore we do not know if the effects observed after addition of PGE_1 to fat tissue are related to the fat cells proper or to other cells

When lipolysis was submaximally stimulated with noradrenaline PGE_1 only inhibited the increased glycerol release in 5 of 10 dogs If catecholamines stimulate lipolysis in dogs by stimulating the activity of the adenyl cyclase system as they are believed to do in rat adipose tissue (Butcher *et al* 1965) this finding suggests that the actions of PGE_1 in dog omental fat are not entirely linked to the formation and accumulation of cyclic AMP as we presently envisage this system (Butcher and Baird 1969) This suggestion is further supported by the unexpected finding that PGE_1 in vitro increased basal glycerol output from the dog fat in some instances and that this effect appeared to be coupled to the unresponsiveness towards PGE_1 when lipolysis was enhanced by catecholamines

It should be mentioned in this connection that there is not only a difference between the in vivo and in vitro effects of PGE_1 on basal lipolysis in fasted dogs but also with regard to noradrenaline stimulated In vivo we have always seen a prompt lowering of the noradrenaline raised levels of FFA and glycerol in plasma when PGE_1 has been infused (Bergström *et al* 1964 Bergstrom *et al* 1966 a)

Comparison of effects of nicotinic acid and PGE_1

Although the responses to nicotinic acid and PGE_1 were not identical there were some striking similarities Thus in the 4 dogs where 0 1 μg of PGE_1 lowered basal lipolysis nicotinic acid did the same Furthermore in the 5 dogs where PGE_1 did not inhibit noradrenaline stimulated lipolysis nicotinic acid was also without effect This suggests that common factors might be involved in the variation in response of omental fat tissue from dog to dog

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References

AULICH, A K, STOCK and E WESTERMANN, Lipolytic effects of cyclic-adenosine 3 5' monophosphate and its butyryl derivatives in vitro, and their inhibition by α- and β adrenolytics *Life Sci* 1967 *6* 929—938

BERGSTROM, S, L A CARLSON and L ORO, Effect of prostaglandins on catecholamine induced changes in the free fatty acids of plasma and in blood pressure in the dog *Acta physiol scand* 1964 *60* 170—180

BERGSTROM, S, L A CARLSON and L ORO, Effect of prostaglandin E_1 on plasma free fatty acids and blood glucose in the dog *Acta physiol scand* 1966 a *67*. 141—151

BERGSTROM, S, L A CARLSON and L ORO, Effect of different doses of PGE_1 on free fatty acids of plasma, blood glucose and heart rate in the nonanesthetized dog *Acta physiol scand* 1966 b *67* 185—193

BERGSTROM, [illegible], L A CARLSON and J R WEEKS, The prostaglandins a family of biologically [illegible]

[illegible] t of nicotinic acid on ACTH and noradrenaline [illegible] studies including comparison with prostaglandin [illegible]

BUTCHER, R W and C E BAIRD, The regulation of cyclic AMP and lipolysis in adipose tissue by hormones and other agents Advances in experimental medicine and biology Vol 4 *Drugs affecting lipid metabolism* (W L Holmes, L A Carlson and R Paoletti ed) Plenum, New York 1969 5—24

BUTCHER, R W C E BAIRD and E W SUTHERLAND, Effects of lipolytic and antilipolytic substances on adenosine 3'5'-monophosphate levels in isolated fat cells *J biol Chem* 1968 *243* 1705—1712

BUTCHER, R W R J HO, H C MENG and E W SUTHERLAND, Adenosine 3'5' monophosphate in biological materials II The measurement of adenosine 3'5'-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine *J biol Chem* 1965 *240* 4515—4523

CARLSON, L A, Studies on the effect of nicotinic acid on catecholamine stimulated lipolysis in adipose tissue in vitro *Acta med scand* 1963 *173* 719—722

CARLSON L A Inhibition of the mobilization of free fatty acids from adipose tissue *Ann N Y Acad Sci* 1965 *131* 119—142

CARLSON, L A Cardiovascular and metabolic effects of prostaglandins *Progr Biochem Pharmacol* 1967 *3* 94—109

[C]ARLSON, L A and A HANNGREN, Initial distribution in mice of ^3H labelled nicotinic acid [s]tudied with autoradiography *Life Sci* 1964 3 867—871

CARLSON L A and H MICHELI *Paradoxical effect of prostaglandin E_1 in vitro on lipolysis in adipose tissue of diabetic rats* In Östman J Ed, Progr VIth Internat Congr Diab Fed Elsevier 1969 In press

CARLSON L A and E R NYE Acute effects of nicotinic acid in the rat I Plasma and liver lipids and blood glucose *Acta med scand* 1966 *179* 453—461

CARLSON, L A and L ORO The effect of nicotinic acid on the plasma free fatty acids Demonstration of a metabolic type of sympathicolysis *Acta med scand* 1962 *172* 641—645

CARLSON L A, S O LILJEDAHL and C WIRSEN Blood and tissue changes in the dog during and after excessive free fatty acid mobilization A biochemical and morphological study *Acta med scand* 1965 *178* 81 102

CARLSON L A, S O LILJEDAHL M VERDY and C WIRSEN, Unresponsiveness to the lipid mobilizing action of catecholamines in vivo and in vitro in the domestic fowl *Metabolism* 1964 *13* 227—231

CARLSON, L A C HEDBOM E HELGSTRAND B SJOBERG and N STJERNSTROM Pyridines affecting FFA mobilization Advances in experimental medicine and biology Vol 4 *Drugs affecting lipid metabolism* W L Holmes, L A Carlson and R Paoletti ed) Plenum New York 1969 85—92

MICHELI, H, Some characteristics of lipolysis in rabbit adipose tissue effect of noradrenaline, ACTH, theophylline and prostaglandin E_1 *Acta physiol scand* 1970 *79* 289 298

MICHELI, H L A CARLSON and D HALLBERG Comparison of lipolysis in human subcutaneous and omental adipose tissue with regard to effects of noradrenaline, theophylline prostaglandin E_1 and age *Acta chir scand* 1969 In press

RUDMAN, D and M DI GIROLAMO Comparative studies on the physiology of adipose tissue In *Advances in Lipid Research* Vol 5 (R Paoletti and D Kritchevesky ed) Academic Press New York and London 1967 35 117

SNEDECOR, G W, *Statistical methods* The Iowa State Univ Press Ames Iowa 1956

STOCK, K and E WESTERMANN Hemmung der Lipolyse durch α und β sympathicolytica Nicotinsäure und Prostaglandin E_1 *Arch Pharmak exp Path* 1966 *254* 334 351

Acta physiol. scand 1970. 79. 153—157

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Effect of Nicotinic Acid on ACTH and Noradrenaline Stimulated Lipolysis in the Rabbit. I. In Vivo Studies

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Abstract

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ACTH 50 10 2 and 0.5 IU/kg b.w and noradrenaline 1.0 μg/kg/min were given i.v. to fed unanesthetized rabbits either pretreated with nicotinic acid or not.

Both ACTH and noradrenaline increased arterial concentration of free fatty acids (FFA). For noradrenaline this effect was present only in rabbits with lower body weight and furthermore the effect was less pronounced and of shorter duration than for ACTH.

Nicotinic acid inhibited partly the increase of plasma FFA caused by 0.5 IU of ACTH but not that after the higher doses. Nicotinic acid had sometimes but not always an inhibitory effect on the increase caused by noradrenaline while nicotinic acid by itself did not change the basal levels of plasma FFA.

Hoak *et al* (1963) demonstrated widespread thrombosis after injection of ATCH in the rabbit 50 IU/kg and suggested that the thrombosis could be due to the excessive mobilization of free fatty acids (FFA) and not to other actions of ACTH. In order to further test this hypothesis we intended to use quantitative methods to measure the intravascular coagulation and to study whether suppression of the ACTH stimulated FFA mobilization had any effect on the blood coagulation. Since nicotinic acid is a well documented inhibitor of FFA mobilization in species as rat dog and man (Carlson and Oro 1962 Carlsson and Bally 1965 and Carlson 1963) this drug was chosen as inhibitor. To our surprise however no inhibition of the FFA mobilization occurred in preliminary experiments in the rabbit. These results stimulated us to further investigate FFA mobilization from adipose tissue in the rabbit and the inhibitory properties of nicotinic acid on this phenomenon.

TABLE I Plasma FFA concentration (mmol/l, mean ± s.e.m.) after administration of different doses of ACTH, with and without nicotinic acid (Nic) pretreatment (200 mg/kg b.w.) Animal weight 2120—3000 g

Treatment	Number of animals	0 min	30 min	60 min	120 min
ACTH 50 IU/kg	3	0.58±0.22	—	2.56±0.20	2.85±0.36
„ + Nic	3	0.50±0.11	—	2.65±0.25	3.09±0.19
ACTH 10 IU/kg	5	0.56±0.08	2.63±0.37	3.32±0.61	2.67±0.37
„ + Nic	6	0.68±0.18	2.04±0.32	2.68±0.28	2.51±0.06
ACTH 2 IU/kg	4	0.49±0.08	2.13±0.20	2.24±0.24	1.42±0.36
„ + Nic	4	0.73±0.22	2.44±0.24	2.65±0.26	1.47±0.33
ACTH 0.5 IU/kg	4	0.48±0.02	2.46±0.53	2.08±0.30	1.07±0.23
„ + Nic	4	0.77±0.11	1.75±0.28*	1.06±0.28**	0.52±0.08***
0.9 % NaCl	4	0.55±0.09	0.69±0.06	0.73±0.06	0.60±0.08
Nic 200 mg/kg	4	0.41±0.02	0.46±0.04	0.46±0.04	0.39±0.06

* $0.02 < p < 0.05$
** $0.001 < p < 0.01$
*** $0.01 < p < 0.02$ for the statistical significance of the effect of nicotinic acid. This was calculated on the individual changes from zero time.

Material and methods

Experimental animals. 47 albino rabbits of both sexes, 1840—3000 g, were used. All animals had free access to food (Ewos pellets) and water. The experiments were carried out at 9 a.m. on free fed unanesthetized animals. During the experiments the rabbits were kept in cages with their heads outside the cage. Their appearance was normal and they did not seem to be upset by the experiment.

Determination of FFA was made according to the method of Dole (1956).

Drugs and injection procedures. ACTH (Cortrophin® Pharmacia AB, Uppsala, Sweden) was injected into a marginal ear vein during one minute. Doses used were 50, 10, 2 and 0.5 IU per kg b.w.

Noradrenaline (Nor Exadrin®, AB Astra, Sodertalje, Sweden) was given as a constant infusion via a polyethylene catheter into a marginal ear vein, 1.0 µg per kg and min during 30 min. The constant infusions were performed with a Braun Unita IIB infusion pump and siliconized precision ground glass syringes.

Nicotinic acid (ACO, Stockholm, Sweden), 200 mg per kg b.w., was given as a single injection during 2 min into a marginal ear vein. The injections of nicotinic acid were always given 5 min before the administrations of ACTH or noradrenaline.

Blood sampling was done by repeated puncture (with a siliconized needle attached to a short polyethylene tubing) of the central artery of the ear. Samples were taken at 0, 30, 60 and 120 min after ACTH injection and at 0, 20, 30, 40 and 60 min after the injections of noradrenaline. Each time 3 ml of blood was taken into heparinized test tubes and after centrifugation the plasma was kept frozen at −20° C until analysed (within 3 weeks).

Results

Iv injection of ACTH caused a pronounced increase of plasma FFA concentration in the rabbit (Table I). A level of between 2.0 and 3.4 mmol/l was reached already 30 min after the ACTH injection in all treated rabbits independent of their body weight. This level was persistent for at least 120 min after ACTH doses of 50

TABLE II Infusion of noradrenaline from 0 to 30 min (1 μg/kg and min) Plasma FFA concentrations (mmol/l) in six animals treated with noradrenaline alone and six animals treated with noradrenaline ± nicotinic acid (200 mg kg) Nicotinic acid given i v 5 min before noradrenaline infusion was started

Treatment	Rabbit no	Body weight in g	0 min	20 min	30 min	40 min	60 min
Noradrenaline	1	1840	0 49	0 90	1 05	0 73	0 70
	2	2230	0 59	0 79	0 94	0 55	0 51
	3	2250	0 51	0 68	0 70	0 90	0 67
	4	2300	0 89	1 60	1 36	0 52	0 51
	5	2430	0 44	0 53	0 45	0 40	0 31
	6	2700	0 75	0 71	0 76	0 57	0 53
Nicotinic acid	7	1750	—	0 28	0 30	0 35	0 37
+	8	1850	0 62	0 64	0 67	0 38	0 37
Noradrenaline	9	2200	0 33	—	0 44	0 37	0 44
	10	2400	0 41	0 45	0 37	0 43	0 37
	11	2470	0 44	0 40	0 38	0 39	0 35
	12	2470	0 39	0 59	0 50	0 45	0 42

and 10 IU/kg body weight even if the animals had been pretreated with nicotinic acid However, in the rabbits injected with 2 and 0 5 IU of ACTH b w the FFA concentration had decreased to 1 5 and 1 1 mmol/l respectively 120 min after the ACTH injection The pretreatment with nicotinic acid had no effect on the FFA concentration of the rabbits receiving 2 IU of ACTH/kg b w or more On the other hand in rabbits receiving 0 5 IU/kg b w the FFA concentration was significantly lower after nicotinic acid pretreatment Nicotinic acid alone did not change the plasma concentration of FFA In the control animals where saline was injected there was a slight increase of plasma FFA concentration but this was not significant

The results from the rabbits infused with noradrenaline are given in Table II In four animals, weighing between 1840 and 2300 g there was a consistent increase of plasma FFA concentration during the infusion However, in two rabbits with body weights of 2400 and 2700 g the FFA concentration was unchanged during the time studied With nicotinic acid pretreatment the noradrenaline stimulated increase in plasma FFA varied In rabbit number 9 and 12 there was an increase but in the rest the stimulatory effect of noradrenaline failed to appear

Discussion

The results of this investigation have shown that ACTH given i v in doses from 0 5 to 50 IU/kg b w increased the arterial concentration of plasma FFA in the rabbit This was well known from earlier reports recently reviewed by Rudman and Di Girolamo (1967) This increase of plasma FFA concentration induced by ACTH

is mainly due to increased mobilization of FFA from adipose tissue as demonstrated by Hirsch *et al* (1963) who showed a negative a v difference of FFA over the peri renal adipose tissue A dose of 50 IU was used by us because this was the dose used by Hoak *et al* (1963) The dose response of ACTH induced increase of FFA mobilization in the rabbit after i v administration has been studied by Lebovitz *et al* (1965) From our results it is evident that the doses 50 10 2 and 0 5 IU/kg body weight give the same maximal level of plasma FFA probably due to a maximum stimulation of lipolysis However, the duration of the effect decreased with the two lowest doses The maximum stimulated mobilization of FFA found after the three *higher doses of ACTH was not affected by nicotinic acid but the effect of 0 5 IU/kg* of ACTH was partly inhibited by nicotinic acid The failure of nicotinic acid to inhibit the rise in FFA seen after 50 IU of ACTH/kg which observation was the stimulus for this study, is probably related to the fact that this dose appears to be more than 10 times higher than the one required to give maximal lipolytic stimulus This suggests a competetive type of inhibition of nicotinic acid on the ACTH effect of FFA mobilization

The question if noradrenaline has any adipokinetic effect in the rabbit has been discussed Rudman *et al* (1963) found no effect 90 min after 1 mg noradrenaline injected s c However Himms Hagen and Hagen (1962), Svedmyr (1966) and Dzedin *et al* (1968) showed that plasma FFA concentration was increased by nor adrenaline We also found that plasma FFA increased during constant infusion of noradrenaline but only in young rabbits, *i e* in rabbits with low body weight This was true even in in vitro studies on adipose tissue in rabbits (Micheli 1969) A lower responsiveness of adipose tissue lipolysis in vitro by noradrenaline in old animals has been shown in other species too *e g* the rat (Jelinkova and Hruza (1964)

Nicotonic acid inhibited completely the noradrenaline induced stimulation of FFA mobilization in some animals The variability in the results with noradrenaline and nicotinic acid was also seen during in vitro studies on rabbit adipose tissue (Boberg *et al* 1969) where nicotinic acid inhibited noradrenaline induced *stimulation in 3* rabbits and had no inhibitory action in 5 other animals Dzedin *et al* (1968) found in rabbits fasted for 16 to 18 hrs that nicotinic acid did inhibit the effect of nor adrenaline on FFA mobilization These authors also found that nicotinic acid alone lowered plasma FFA concentration in the rabbit Nicotinic acid alone did not de crease the plasma FFA concentration in our study where fed rabbits were used However their fasting FFA levels were about twice as high as ours

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References

Boberg J H Micheli and L. Rammer Effect of nicotinic acid on ACTH and noradrenaline stimulated lipolysis in the rabbit II In vitro studies including comparison with prostaglandin E_1 *Acta physiol scand* 1970 *79* 299—304

Carlson L A, Inhibition of the mobilization of free fatty acids from adipose tissue *Ann N Y Acad Sci* 1965 *131* 119—142

Carlson L A and P R Bally Inhibition of lipid mobilization In *Handbook of Physiology Section 5 Adipose Tissue* American Physiological Society, Washington 1965 p 557—574

Carlson L A and L Oro The effect of nicotinic acid on the plasma free fatty acids. Demonstration of a metabolic type of sympathicolysis *Acta med scand* 1962 *172* 641—645

Dole V P A relation between non-esterified fatty acids in plasma and the metabolism of glucose *J clin Invest* 1956 *35* 150—154

Dzedin T L Lundholm and N Svedmyr Relationship between some metabolic effects of nicotinic acid and catecholamines in the rabbit *Acta pharmacol* (Kbh) 1968 *26* 446—460

Himms Hagen J and P B Hagen An enzymic method for estimation of glycerol in blood and its use to determine the effect of noradrenaline on the concentration of glycerol in blood *Can J Biochem Physiol* 1962 *40* 1129—1139

Hirsch R L D Rudman R Ireland and R K Seraly Movement of the free fatty acids into and out of the blood stream in normal rabbits and in rabbits injected subcutaneously with pituitary adipokinetic substances Fraction H and adreno-corticotropin *J Lipid Res* 1963 *4* 289—296

Hoak J C J C F Poole and Ph D Robinsson Thrombosis associated with mobilization of fatty acids *Amer J Path* 1963 *43* 987—998

Jelinkova, M and Z Hruza Decreased effect of norepinephrine and growth hormone on the release of free fatty acids in old rats *Physiologia bohemoslov* 1964 *13* 327—332

Lebovitz H E, K Bryant and L A Frohman Acute effects corticotrophin and related peptides on carbohydrate and lipid metabolism *Ann N Y Acad Sci* 1965 *131* 274—287

tissue effect of noradrenaline
d 1970 *79* 145—152
ne physiology of adipose tissue
D Kritchevsky Edit, Academic

The influence of thyroxine treatment and thyroidectomy on the calorigenic and some other metabolic effects of adrenaline and noradrenaline in experiments on fasted rabbits *Acta physiol scand* 1966 *66* 257—268

Acta physiol. scand. 1970. 79. 158—166

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The Influence of an Increased Intracranial Pressure on the Lactate, Pyruvate, Bicarbonate, Phosphocreatine, ATP, ADP and AMP Concentrations of the Cerebral Cortex of Dogs

By

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Abstract

ZWETNOW, N N *The influence of an increased intracranial pressure on the lactate, pyruvate, bicarbonate, phosphocreatine, ATP, ADP and AMP concentrations of the cerebral cortex of dogs* Acta physiol scand 1970 79 158—166

In order to study effects of increased cerebrospinal fluid pressure upon brain tissue concentrations of bicarbonate, lactate pyruvate ATP, ADP, AMP and phosphocreatine the metabolites were measured in cortical tissue of 37 dogs frozen in situ during and after a 15 min decrease of the cerebral perfusion pressure to 30—35 mm Hg induced by connecting the cisterna magna to a reservoir with artificial CSF The intracranial hypertension was found to give a variable but marked increase in tissue lactate and pyruvate, and in the lactate/pyruvate ratio as well as a corresponding decrease in the bicarbonate concentration Marked decreases in the ATP and phosphocreatine concentrations were also seen Resting values for these metabolites were not approached until 30—90 min after restitution of normal perfusion pressure Since the changes in ATP and phosphocreatine corresponded in time to the changes in the lactate pyruvate and bicarbonate concentrations it is concluded that the decrease in the cerebral perfusion pressure gave rise to a longlasting disturbance of the oxidative metabolism possible due to reversible structural tissue damage Cerebral blood flow measured in 10 of the dogs showed varying decreases of cerebral blood flow during the intracranial hypertension period Usually hyperemia of 30—90 min duration followed when the CSF pressure was released The hyperemia and the metabolic changes were of approximately the same duration

We have recently studied the effects of an increased cerebrospinal fluid (CSF) pressure on the cerebral circulation (Haggendal *et al* 1970 a and b) and on acid base parameters and lactate and pyruvate concentrations in cerebral venous blood and cisternal CSF (Kjallquist *et al* 1969 a and b) In one of those papers (Haggendal *et al* 1970 b) it was shown that when the cerebral perfusion pressure was reduced to about 40 mm Hg or lower restitution of a normal perfusion pressure was almost invariably followed by a period of increased cerebral blood flow ("reactive hyperemia"), which usually lasted for a quarter of an hour to several hours In another paper we could show that such reductions of the perfusion pressure were accompanied

by an increased lactate concentration and by an increased lactate/pyruvate ratio in cerebral venous blood (Kjällquist *et al* 1969 a) Once a normal perfusion pressure had been restored there were no lactate and pyruvate changes in the cerebral venous blood, indicative of a continuous lactate production in the tissue but analyses on cisternal CSF showed an increased lactate concentration and an increased lactate/pyruvate ratio, which could persist for hours (Kjällquist *et al* 1969 b)

The main object of the present paper was to study if the longlasting extracellular acidosis and the longlasting increase in the CSF lactate/pyruvate ratio was due to a slow clearing of lactate from the CSF, or if they were due to a continuous lactate production in a tissue the oxidative metabolism of which was disturbed for long periods Analyses were therefore made of lactate pyruvate ATP, ADP, AMP, phosphocreatine and bicarbonate concentrations of cortical tissue of dogs frozen *in situ* after a standardized decrease of the cerebral perfusion pressure to 30—35 mm Hg for a period of 15 minutes A preliminary communication on a part of the present results has been given earlier (Zwetnow 1968)

Methods

The present results are based on experiments in 35 mongrel dogs which were anesthetized with sodium pentobarbital (Nembutal Abbott) in an initial dose of 50 mg/kg supplemented with repeated small doses at intervals The animals were intubated and ventilated artificially with a Starling type respirator Immobilization was achieved with i v suxamethonium chloride (Celocurin Klorid Vitrum) The ventilation was set with the intention of keeping the P_{aCO_2} as close to 40 mm Hg as possible The rectal temperature was measured with a mercury thermometer, and the temperature of the animal was kept as close to 37° C as possible by means of intermittent heating or cooling One femoral artery was cannulated for blood pressure recording with an electromanometer (Elema Stockholm) and for sampling of arterial blood and one femoral vein was cannulated for injections The left vertebral artery was cannulated fo [illegible]

[illegible] double barrelled needle which permitted continuous measurements of the cisternal pressure For details of technique and experimental procedures reference is made to the previous publications (Häggendal *et al* 1969 a and b see also Kjällquist *et al* 1969 a and b)

Arterial blood was analyzed for pH P_{CO_2} and for the oxygen saturation In some animals cerebral venous blood was sampled from the superior sagittal sinus and analyzed for pH P_{CO_2} and oxygen saturation

For the freezing of the tissue *in situ* the parietal cortex was exposed on one side via a craniotomy but the dura was always left intact and care was taken not to press on the tissue A plastic funnel was fitted into the skin incision over the craniotomy for subsequent freezing of the tissue The tissue was frozen by pouring liquid nitrogen into the funnel (Pontén 1966 a The freezing was continued for at least 30 min during which time the blood pressure was upheld at normal values After the end of the freezing period the subdural cortical tissue was chiselled [illegible] The tissue which was taken for subse[illegible] he region of the craniotomy which was [illegible] sphocreatine lactate and pyruvate were [illegible] ng extraction of the tissue at −10° C (Lowry *et al* 1964) neutralization of the perchloric acid extracts with individual electrometric pH control and recording of each enzyme reaction on a lin log recorder (see Granholm *et al* 1968)

The total CO_2 content of the tissue was analysed with the method described by Pontén and Siesjö (1964) The values given in the text are mean values of at least 3 individual determinations on each brain sample The mean bicarbonate concentration of the tissue was calculated by subtracting the physically dissolved CO_2 from the total CO_2 content determined.

The physically dissolved CO_2 was calculated as the product of the mean tissue CO_2 tension (P_{tCO_2}) and the solubility factor 0 0292 (Siesjo 1962) The mean tissue CO_2 tension was derived from the relations given by Ponten and Siesjo (1966) This calculation will markedly underestimate the tissue CO_2 tension, and overestimate the "tissue pH", in those few animals which were studied during the intracranial hypertension However, also in the animals studied in the reactive hyperemia phase, the calculation will slightly overestimate the tissue CO_2 tension but the effect on the bicarbonate concentrations will be insignificant The mean bicarbonate concentration was calculated for the water phase of the tissue and no attempt was made to partition the bicarbonate (or the lactate and pyruvate) between extracellular and intracellular spaces However, since the P_{tCO_2} varied between the individual animals a calculation of the base excess values was made

For the calculation of the base excess values of the tissue the buffer curve published by Pontén (1966 b) for a rat brain tissue was used, while the 'tissue pH' was calculated from the equation

$$pH_t = 6\ 120 + \log \frac{(T_{CO_2} - P_{tCO_2}\ 0\ 0292)}{P_{tCO_2}\ 0\ 0314\ 0\ 78}$$

(compare Kjallquist Nardini and Siesjo 1969)

Results

The present results were obtained in experiments in which the cerebral perfusion pressure could be adequately maintained at 30—35 mm Hg for 15 min In a few experiments the perfusion pressure increased during the pressure period due to a Cushing effect, and these experiments were therefore excluded from the material In 2 expts the perfusion pressure was lower (15—20 and 0 mm Hg respectively) and the results obtained in these experiments were separatively indicated In some experiments the tissue were extracted at 0° C, and not at −10° C, whence the analyses of the phosphocreatine ATP, ADP and AMP concentrations were discarded (see Granholm *et al* 1968)

In one part of the experiments CBF measurements were performed and could thus be used to show the effect of the induced rise in CSF pressure on the cerebral circulation In the remaining animals the induced intracranial hypertension could be verified either by significant changes in the EEG or by a reduction in the cerebral venous oxygen saturation In all animals moreover, a fluid seeping from the nostrils was observed during the intracranial hypertension period

Seven animals served as controls In 6 dogs the cerebral tissue samples were taken about 1—1 5 hr after the initial anesthesia In the seventh dog however 5 hrs were

TABLE I Acid base parameters and metabolites in arterial blood and in cerebral cortical tissue of dogs anesthetized with pentobarbital M±S D The ranges are given in the figures Substrate concentrations in meq/kg Number of expts within parentheses

Arterial blood			Cerebral cortical tissue								
SaO_2 %	P_{CO_2} mm Hg	pH	La	Py	La / Py	PCr	ATP	ADP	AMP	TCO_2	Tissue "pH
90(7) ±4	39(7) ±6	7 36(7) ±0 03	0 93(7) ±0 07	0 059(7) 0 003	16 1(7) ±1 5	4 08(5) ±0 26	2 58(5) ±0 07	0 33(5) ±0 07	0 09(5) ±0 05	14 43(5) ±1 33	7 19(5) ±0 01

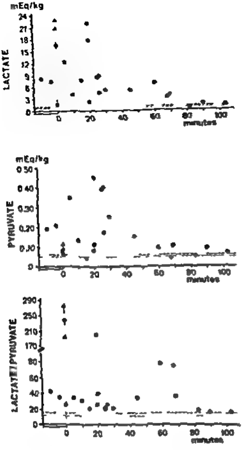


Fig 1

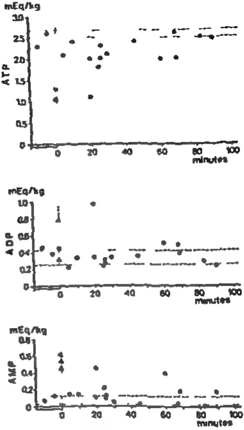


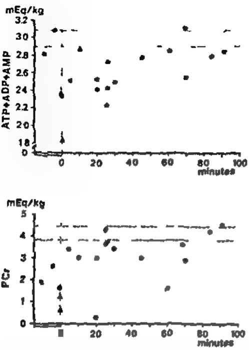
Fig 2

brain tissue during
35 mm Hg (filled
mm Hg Horizontal

Fig 2 ATP ADP and AMP concentrations in brain tissue during and after a 15 min period of lowered cerebral perfusion pressure to 30—35 mm Hg (filled circles) Filled triangles represent does with a perfusion pressure below 30 mm Hg Horizontal dotted lines indicate the range of control values

allowed to pass before the tissue sampling but this did not affect the concentration of the metabolites measured The mean values of the control group are listed in Table I

Fig 1 shows the lactate and pyruvate concentrations measured and the lactate/pyruvate ratios calculated for the brains frozen during and after the period of increased intracranial pressure The figure shows that the lactate concentration increased from the normal values of around 1 mmoles/kg tissue to values sometimes exceeding 20 mmoles/kg but the increase in the lactate concentration varied appreciably between the individual animals The results clearly showed however that the lactate concentration of the tissue remained increased for long periods after a normal perfusion pressure had been restored and normal or near normal values were not



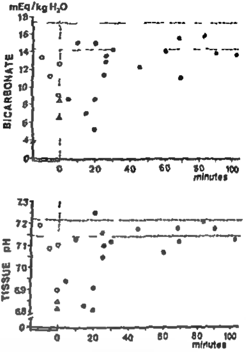


Fig 3 Fig 4

Fig 3 The sums of adenine nucleotide concentrations and the phosphocreatine concentrations in brain tissue during and after a 15 min period of lowered cerebral perfusion pressure to 30—35 mm Hg (filled circles) Filled triangles represent dogs with a perfusion pressure below 30 mm Hg Horizontal dotted lines indicate the range of control values

Fig 4 Bicarbonate concentrations and calculated tissue pH in brain tissue Unfilled circles represent values during a 15 min period of cerebral perfusion pressure reduced to 30—35 mm Hg Underestimations of tissue pCO_2 in these animals give correspondingly overestimated values of bicarbonate and tissue pH Filled circles represent animals after normalization of perfusion pressure

obtained until 1—1 5 hr after the end of the pressure period A similar increase was seen in the tissue pyruvate concentrations but since the lactate concentrations were proportionally higher than the pyruvate concentrations the increase in the lactate/pyruvate ratios resembled the increase in the lactate concentrations

The tissue concentrations of ATP ADP and AMP are shown in Fig 2 There were rather small and inconsistent variations in the ADP and AMP concentrations but a marked and longlasting decrease in the ATP concentrations and control values for ATP were not reached or approached until 60—90 min after the restoration of a normal intracranial pressure The decrease in the ATP concentrations were reflected in the sum of the adenine nucleotides which are shown in Fig 3 together with the phosphocreatine concentrations The latter varied in a similar way to the ATP concentrations in showing both a wide scatter between the individual experiments and a slow restitution

The increase in the tissue lactate concentration (see above) was parallelled by a decrease in the tissue bicarbonate concentration and in the calculated tissue pH (Fig 4) It can be seen that bicarbonate values as low as 6 meq/kg of tissue water

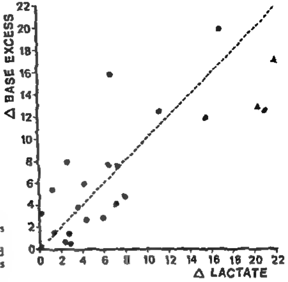


Fig 5 Relation between calculated increase in the negative base excess values and in the lactate concentrations in the same animals as depicted in Fig 1 and 4 Dotted line represents line of equality

were obtained in the immediate post pressure period and that the calculated pH was decreased by maximally about 0 4 units An even greater fall in bicarbonate and pH should have occurred during the pressure period since the values given (unfilled symbols) represent overestimations of the bicarbonate and of the actual pH values due to an unrecorded increase in the tissue CO_2 tension during the period of increased intracranial pressure

In order to correct for changes in the CO_2 tension between the individual animals the calculated increase in the negative base excess values have been compared to the increase in the lactate concentrations in Fig 5 The figure suggests a one-to-one relationship between the increase in lactate, and the decrease in the negative base excess, although a fairly wide scatter precludes too rigid an interpretation

The cerebral cortical blood flow was measured in 10 animals. Fig 6 shows the cortical blood flow measured as described in Methods but here expressed in per cent of the initial flow value Despite a certain variation in absolute flow values the majority of animals showed a rather similar flow response, i e a flow reduction during the period of intracranial hypertension and a hyperemia upon restoration of the intracranial pressure to normal All 10 animals showed decreased cortical blood flow values (varying between 3 and 50 % of initial control flow) during the pressure period In 2 of the animals, however the flow reduction was not significant A sig- …

… of 6 animals with an adequate number of CBF values the hyperemia could be seen to be completely or partially reversible The duration varied between 30 and 90 minutes with an average figure of about 50 min

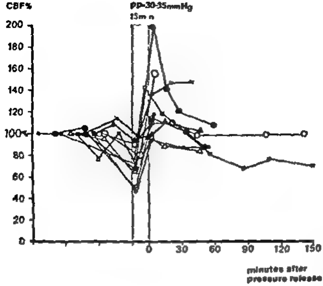


Fig. 6. Cerebral cortical blood flow before, during and after a perfusion pressure decrease to 30—35 mm Hg for 15 min. Figures are given in % of normal (here denoted as 100 %).

Discussion

The present results have shown that a decrease in the cerebral perfusion pressure to 30—35 mm Hg which is known to give rise to a period of reactive hyperemia (Hagendal *et al.* 1969 b) and which is known to be accompanied by a long-lasting extracellular lactacidosis with an increased lactate/pyruvate ratio (Kjällquist *et al.* 1969 b), equally gave rise to a long-lasting and marked lactacidosis in the brain tissue proper. This lactacidosis was characterized by an increased lactate concentration, a correspondingly decreased bicarbonate concentration, and by an increased lactate/pyruvate ratio. Thus, although there was a marked variability in the degree of the lactacidosis in the tissue, probably in part due to undetected variations in the existing perfusion pressures, the results show that normal lactate concentrations, and normal lactate/pyruvate ratios were not approached before at least one hour had passed after the restoration of a normal perfusion pressure. The assumption that the variability in the results between the individual experiments was due to variations in the effective perfusion pressure was strengthened by the high lactate values and the high lactate/pyruvate ratios obtained in those experiments in which the perfusion pressure was decreased to values below 30 mm Hg (see Fig. 1), and also by the fact that the animals with the highest lactate values had low phosphocreatine and ATP values.

It seems logical to assume that the increase in the tissue lactate concentration was due to a reduced blood flow, causing a tissue hypoxia with an increased anaerobic glucolysis. Thus, it is known that hypoxia, whether induced by low oxygen concentrations in the inspired air or by interruption of blood supply to the tissue, is accompanied by a decrease in the tissue concentrations of phosphocreatine and ATP, and

by corresponding increases in the concentrations of ADP, AMP and lactic acid (Gurdjian et al 1944, Klein and Olsen 1947, Lowry et al 1964, Thorn et al 1958, Schmahl et al 1965)

However, although the present results show that the type of tissue hypoxia produced by intracranial hypertension gives metabolic changes similar to those observed after other types of hypoxia, the slow or imperfect restitution of the changes is striking

Recent experiments from this laboratory have shown that if rats are asphyxiated for 3 min virtually all phosphocreatine in the brain is broken down, and the intracellular lactate concentration approaches 25 mMoles/kg of water (Kaasik, Nilsson and Siesjo 1969 a) However, in spite of these marked metabolic changes, a normal metabolite pattern is seen 10 min after the resumption of normal ventilation These results are in sharp contrast to the present ones which show that if the cerebral perfusion pressure is decreased in dogs to 30—35 mm Hg for 15 min by means of an increase in the CSF pressure, resting values for ATP and phosphocreatine are not obtained until 30—90 min after the restitution of a normal perfusion pressure Taken in conjunction with the lactate, pyruvate and bicarbonate concentrations measured under the same conditions, it would appear as if the increased intracranial pressure gave rise to a condition of tissue hypoxia the effects of which only slowly disappear (cf Kaasik et al 1969 b) This would indicate that a reduced cerebral blood flow induces a reversible structural tissue damage or a condition in which some part of the tissue is underperfused for long periods

There was a striking variation in the metabolic pattern of the individual dogs during and after the period of increased intracranial pressure One reason for this variation is probably critical variations in the perfusion pressure which are not resolved by the methods used to measure the arterial and the CSF pressures It is possible that more continuous measurements of the cerebral blood flow during the pressure periods would have disclosed differences in the actual tissue perfusion, responsible for the metabolic changes observed The variation in flow reduction between the dogs, of which two had a near normal cortical flow, confirm earlier findings (Haggendal et al 1969 b) which indicate that a cerebral perfusion pressure of 30—35 mm Hg is just below the limit of the cerebral autoregulatory ability It has also been shown that if the perfusion pressure excess 40 mm Hg there are no changes in the tissue concentrations of phosphocreatine ATP ADP and AMP (Reulen et al 1967, Siesjo and Zwetnow 1969) These facts thus strongly suggest that the present tissue changes are due to a marked but variable fall in the cerebral blood flow The variability in the flow reduction at these marginal perfusion pressures may also explain the variability in the metabolic changes

Though it seems to be a rough temporal correlation between the changes in the concentrations of lactate pyruvate ATP and phosphocreatine and between changes in the cortical blood flow, the material does not allow any conclusions to be drawn regarding any causal relations between changes in the concentrations of any of the metabolites measured and their possible vasodilatory action during the hyperemic period

References

GRANHOLM, L, A KAASIK, L NILSSON and B K SIESJÖ, The lactate/pyruvate ratios of cerebrospinal fluid of rats and cats related to the lactate/pyruvate the ATP/ADP, and the phosphocreatine/creatine ratios of brain tissue *Acta physiol scand* 1968 74 398—409

GURDJIAN, E S, W E STONE and J E WEBSTER, Cerebral metabolism in hypoxia *Arch Neurol* 1944 51 472—477

HAGGENDAL, E, J LOFGREN, N J NILSSON and N N ZWETNOW, Effects of varied cerebrospinal fluid pressure on cerebral blood flow in dogs *Acta physiol scand* 1970 79 262—271

HAGGENDAL E, J LOFGREN, N J NILSSON and N N ZWETNOW, *Prolonged cerebral hyperemia after periods of increased cerebrospinal fluid pressure in dogs Acta physiol scand* 1970 79 272—279

HAGGENDAL E N J NILSSON and B NORBACK, On the components of Kr^{85} clearance curves from the brain of the dog *Acta physiol scand* 1965 *Suppl 258* 5—25

HOHORST, H J F H KREUZ and T BÜCHER, *Über Metabolitgehalte und Metabolit Konzentrationen in der Leber der Ratte Biochem Z* 1959 *332* 18—46

KAASIK A E L NILSSON and B K SIESJO, The effect of asphyxia upon the lactate, pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970a *78* 433—447

KAASIK A E L NILSSON and B K SIESJO The effect of arterial hypotension upon the lactate, pyruvate and bicarbonate concentrations of the brain tissue and cisternal CSF, and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970b *78* 448—458

KJALLQUIST A M NARDINI and B K SIESJO, The regulation of extra and intracellular acid base parameters in the rat brain during hyper- and hypocapnia *Acta physiol scand* 1969 *76* 485—494

KJALLQUIST A B K SIESJO and N N ZWETNOW Effects of increased intracranial pressure on cerebral blood flow and on cerebral venous pO_2, pCO_2 pH, lactate and pyruvate in dog *Acta physiol scand* 1969 *75* 267—275

KJALLQUIST A B K SIESJO and N N ZWETNOW *Effects of increased intracranial pressure* on cerebral blood flow and on cerebrospinal fluid HCO_3, pH lactate and pyruvate *Acta physiol scand* 1969 *75* 345—352

…LEIN J R and N S OLSEN Effect of convulsive activity upon the concentration of brain glucose glycogen lactate and phosphates *J biol Chem* 1947 *167* 747—756

…ASSEN N A K HOEDT RASMUSSEN S C SORENSEN E SKINHOJ E CRONQUIST S BODFORSS and D H INGVAR Regional cerebral blood flow in man determined by $krypton^{85}$ *Neurology* 1963 *13* 719—727

…OWRY O H I V PASSONEAU F X HASSELBERGER and D W SCHULZ Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain *J biol Chem* 1964 *239* 18 30

…ONTEN U Acid base changes in rat brain tissue during acute respiratory acidosis and baseosis *Acta physiol scand* 1966 a *68* 152—163

…ONTEN U Consecutive acid base changes in blood brain tissue and cerebrospinal fluid during respiratory acidosis and baseosis *Acta neurol scand* 1966 b *42* 455—471

…ONTEN U and B K SIESJO A method for the determination of the total carbon dioxide content of frozen tissues *Acta physiol scand* 1964 *60* 297 308

…ONTEN U and B K SIESJO Gradients of CO_2 tension in the brain *Acta physiol scand* 1966 *67* 129 140

REULEN J J L STELTER W BRENDEL and F MARGUTH Elektrolyt und Metabolit konzentrationen im Gehirn nach normovolämischer Drucksenkung *Z ges exp Med* 1968 *241*—*260*

SCHMAHL F W E BETZ H TALKE and H J HOHORST Energiereiche Phosphate und Metabolite des Energiestoffwechsels in der Grosshirnrinde der Katze *Biochem Z* 1965 *342* 518—531

SIESJÖ B K The solubility of carbon dioxide in cerebral cortical tissue from cats at 37 5° C With a note on the solubility of carbon dioxide in water 0 16 M NaCl and cerebrospinal fluid *Acta physiol scand* 1962 *55* 325—341

SIESJO, B K and N N ZWETNOW The effect of moderate arterial hypotension on acid base parameters and energy metabolites in the rat brain *Acta physiol scand* 1969 *00* 000—000

THORN, W, H SCHOLL G PFLEIDERER and B MUELDENER Stoffwechselvorgänge im Gehirn bei normaler und herabgesetzter Korpertemperatur unter ischämischer und anoxischer Belastung *J Neurochem* 1958 *2* 150 165

ZWETNOW, N N, Effects of intracranial hypotension Acid base changes and lactate changes in CSF and brain tissue *Scand J clin Lab Invest* 1968 Suppl 102 III D

Acta physiol. scand. 1970. 79. 167—173
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The Rising Phase of the Active State in Single Skeletal Muscle Fibres of the Frog

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Abstract

EDMAN, K. A. P. *The rising phase of the active state in single skeletal muscle fibres of the frog.* Acta physiol. scand. 1970. 79. 167—173.

An approach of analyzing the initial phase of the active state in single skeletal muscle fibres of the frog has been designed. The fibre was stimulated to generate a series of four incompletely fused isometric twitches with the object of producing distinct peaks and throughs in the myogram. The peaks and troughs of the records both represent the tension output when the contractile unit was stationary. The tension recorded at these instances therefore provided an adequate measure of the intensity of the active state at respective times after the stimulus. By releasing the fibre at different moments during the third cycle it was possible to define 1. the decay phase of the active state in the third cycle and 2. a substantial portion of the rising phase of the active state in the fourth cycle. It was found that the active state underwent a very rapid increase starting at 12 msec after the stimulus (at 1—2° C). Only 3—4 msec were required for the active state to rise from 25 to 65 per cent of its peak value. The rate of rise of the active state was independent of the sarcomere length (between 2.8 μ and 2.0 μ) and of the degree of activity existing in the fibre when the fourth stimulus was applied. The implications of the results with respect to the kinetics of the calcium activator mechanism in the excitation-contraction coupling are discussed.

According to the analytical model advanced by A. V. Hill (1938) muscle is assumed to function as an active contractile unit in series with a passive elastic element. When the muscle is stimulated the contractile unit is transferred into an 'active state' characterized by capacity to produce tension or motion. Using Hill's analytical approach the intensity of the active state can be experimentally defined as tension or shortening velocity, at selected times during contraction. Evidence has been obtained in recent studies of skeletal muscle (Jewell and Wilkie 1960, Edman and Kiessling 1966) and myocardium (Brady 1966, Edman, Grieve and Nilsson 1966, Edman and Nilsson 1968) that the time course of the active state is not definitely programmed when the contraction starts. The duration of the active state is dependent upon the sarcomere length existing at the onset of contraction and the amount of shortening taking place during the contraction period. For a quantitative analysis of the active state sarcomere length and movement thus have to be adequately controlled.

While the decay phase of the active state and its relation to the isometric myogram has been extensively investigated in previous studies (Wilkie 1956 for earlier references, Jewell and Wilkie 1960, Edman *et al.* 1960) little is known up to the present time about the rising phase of the active state in skeletal muscle. Attempts have been made in studies on whole muscle preparations to define at which time after a single stimulus the contractile material is fully activated. Experiments by Hill (1949), Jewell and Wilkie (1958) and Close (1962) suggest that 40—60 msec are required after the stimulus at 0° C before the muscle is able to carry maximal load. However, in none of the previous studies has it been feasible to evaluate the time course of the active state before the attainment of maximum. As information about the early phase of the active state is of relevance to an understanding of the kinetics of the excitation-contraction coupling the problem has been reinvestigated in isolated muscle fibres. Evidence will be presented that the capacity of the fibre to produce tension undergoes an abrupt increase within a time interval of 12—16 msec after the stimulus.

Methods

Single fibres isolated from the semitendinosus muscle of *R. temporaria* were used. Care was taken to clean the fibre from connective tissue and only a small portion of the original tendons, approximately 2 mm long and 0.5 mm wide, was left at each end of the fibre. The fibre was mounted horizontally in a thermostated (1—2° C) chamber (Edman 1966) between a tension transducer (RCA 5734) and a lever. The rest length and the degree of shortening of the fibre was controlled by micrometer screws in front of and behind the lever. The lever could be quickly released at any selected time during an isometric contraction to allow the fibre to redevelop tension at a shorter pre-set length. The fibre was stimulated by passing current (rectangular pulses, 1 msec duration) through an assembly of 6—8 platinum wire electrodes spaced at 2 mm intervals along the length of the fibre. The electrodes were arranged as alternate anodes and cathodes and care was taken to ensure that each pair of electrodes produced a supramaximal stimulus. The sarcomere length of the fibre at rest was measured by direct microscopy at 800 times magnification as described previously (Edman 1966). A Ringer's solution of the following composition was used (mM): NaCl 115.5, KCl 2.0, $CaCl_2$ 1.8, NaH_2PO_4/Na_2HPO_4 buffer 2.0, pH 7.0.

The term active state is used in the following to denote the capacity of the muscle fibre to produce tension at a given moment. The intensity of the active state is expressed in milligrams or in percent of P_{max}, the tension produced in a completely fused tetanus.

Results

The principle of the analytical approach is illustrated in Fig. 1 and Fig. 2. The fibre was stimulated at 2 min intervals to produce a series of 4 incompletely fused twitches. The stimuli were spaced appropriately so as to obtain distinct peaks and troughs in the isometric myogram (Fig. 1). The peaks and troughs both represent the tension output when the contractile system was stationary, i.e. when the active unit was momentarily in equilibrium with the series elastic component. The tension recorded at these points therefore denotes the capacity of the contractile element to produce tension at these instants after the stimulus (Ritchie 1954). By releasing the fibre at different times after the third stimulus it was possible to derive multiple data on 1) the decay phase of the active state in the third cycle, 2) the rising phase of the active state in the fourth cycle and 3) the decay phase of the activity in the fourth

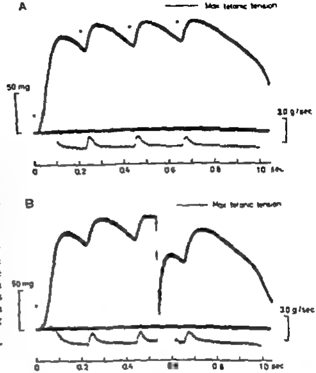
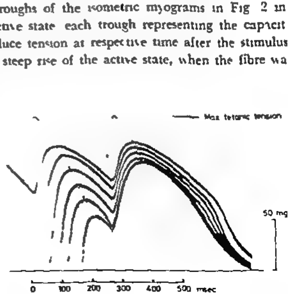


Fig 1 Isometric myograms illustrating four partially summated twitches of single muscle fibre A in the absence and B in the presence of quick release during the third cycle Electrically differentiated signal of the tension transducer displayed below base line Stimulation signals are shown above tension record Note that the trough in the myogram in response to the fourth stimulus is obtained at a different tension level by releasing the fibre during the third cycle Sarcomere length 2 15 μ Amount of release approx 2 5 per cent of the sarcomere length

cycle Fig 1B shows an example of a complete quick release recording and Fig 2 on a faster time base a series of photographically superimposed traces after quic release during the third cycle

The line drawn through the troughs of the isometric myograms in Fig 2 indicates the rising phase of the active state each trough representing the capacit of the contractile element to produce tension at respective time after the stimulus As can be seen there was a very steep rise of the active state, when the fibre wa

Fig 2 Photographically superimposed tension records illustrating third and fourth contraction cycles of a series of 4 incompletely fused twitches Quick release (only redevelopment of tension shown) carried out at different instants during the third cycle Uppermost trace without release Stimulation signals are indicated on top of tension records The troughs and peaks in the myograms provide points on the rising and falling phases of the active state curve (dashed line) Note the rapid onset of the active state Same fibre and sarcomere length as in Fig 1



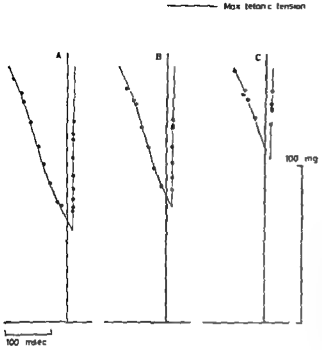


Fig 3 Diagrams illustrating the *rising phase of the active state* initiated from different degrees of decay of preceding active state cycle Time of stimulation indicated by vertical line Analysis carried out as illustrated in Fig 2 Stimulation frequency A 5 per sec, B 6 per sec, C 7 per sec Sarcomere length 2 15 μ

restimulated before the previous activity had ceased The active state started to rise after a latency of about 12 msec Only 3—4 msec were then required for the active state to increase from 25 to 65 per cent of its maximum Fig 3 illustrates the results an experiment in which the stimulation frequency was varied between 5 and 7 per sec In this way the active state was initiated at different levels of relaxation of the fibre It is seen that the same steepness of the rising phase existed irrespective of the degree of activity in the fibre at the moment when the stimulus was applied

Fig 4 shows that the steepness of the initial phase of the active state was not markedly influenced by the sarcomere length Illustrated are active state data obtained from the same fibre at 2 07 μ and 2 77 μ sarcomere lengths respectively It can be seen that the rate of rise of the active state was the same within the limits of resolution of the method in the two situations

The upper-most portion of the active state curve is not accessible for analysis by means of the present approach For this reason it is not possible to assess at which time after the stimulus peak activity is attained (*cf* Hill 1949, Jewell and Wilkie 1958, Close 1962) Nor can it be decided whether the active state does reach the same level as in a fused tetanus under the conditions studied as has been tentatively indicated in Fig 2

The results obtained were probably not influenced by the rapid length changes imposed on the fibre as is evident from the following The quick release was carried out during the contraction cycle *preceding* the cycle under investigation, and at least 100 msec elapsed from the moment of release until the trough occurred in the myo-

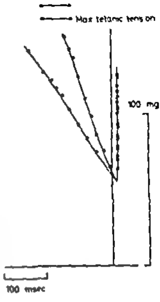


Fig 4 Rising phase of the active state defined at two different sarcomere lengths in the same fibre ○ 2 77 μ stimulation frequency 3 per sec ● 2 07 μ stimulation frequency 5 per sec Analysis carried out as illustrated in Fig 2 The stimulation frequencies were selected so as to obtain approximately the same amount of decay of the active state between stimuli at the two sarcomere lengths

gram (Fig 2) Furthermore, the contractile unit was brought to a momentary standstill after the release as it had to pass through an isometric maximum before the new stimulus was applied On this basis it would seem most unlikely that any effect of the quick release upon the active state should persist during the fourth cycle A further argument in support of this view is the finding that the through value obtained in the myogram without release (top trace Fig 2 falls on the same steep line as the data collected from the records with release

Discussion

The experimental approach used has made it possible to define the time course of the rising phase of the active state in a single muscle fibre The results have shown that there is a very rapid rise of the activity starting at about 12 msec after the stimulus No more than 4 msec are required for the active state to change from 25 to 65 per cent of its maximum value The initial rise of activity in the skeletal muscle fibre is thus considerably faster than suggested by previous studies on whole muscle According to these (for references see above) maximal capacity to produce tension at 0° C is attained 40—60 msec after the stimulus By extrapolation from the present results on the other hand maximal active state would be reached approximately 20 msec after stimulation At present there is no definite explanation of this apparent discrepancy in results It may be that the rate of development of the active state becomes progressively smaller during the upper most portion of the rising phase and that the peak intensity of the active state is approached smoothly (*cf* Close 1962 Wilkie 1966 Taylor 1969) Information on this point could not be obtained in the present analysis

The results provide relevant information about the kinetics of the mechanism that governs the contractile activity in the muscle fibre. If this control function is accomplished by means of calcium, as is now generally held, it is logical to assume that the *active state* reflects the concentration of this agent at the contractile sites. On this basis then the present results can be taken as evidence that the reactive sites in the fibre are saturated to 60—65 per cent with calcium (from a starting level of 20—25 per cent) within 16 msec after the stimulus at the temperature (1—2° C) considered. This rapid activation of the contractile system would seem to require that 1. the source of activator calcium is situated in the immediate vicinity of the individual myofibril and 2. calcium is rapidly released from the storage site when the fibre is stimulated. The first point is adequately fulfilled if calcium is released from the sarcoplasmic reticulum (Sandow 1965). The time needed for the calcium to reach the centre of the individual myofibril under such conditions can be estimated (Hill 1929) by assuming that diffusion occurs into a cylinder of 1 μ diameter (the approximate thickness of the myofibrils) exposed to a constant concentration of calcium from the surface. (In this calculation no account is taken of the binding of calcium to the contractile material.) The diffusion coefficient of calcium in free solution is 7.8×10^{-6} cm²/sec (Wang 1953). A lower value would be expected in the myofibril. However, even if the rate of diffusion inside the myofibril were only 1/50—1/100 of that in free solution, it would take no more than 5—10 msec to raise the concentration of free calcium in the centre of the myofibril to 90 per cent of the concentration in the intermyofibrillar space. It thus seems likely that the transport of calcium from the stores into the contractile system may be achieved rapidly enough to enable the steep rise of the active state.

Attempts have recently been made to record the intracellular kinetics of activator calcium using murexide (Jobsis and O'Connor 1966) and a bioluminescent protein aequorin (Ashley and Ridgway 1968) as indicators. The results obtained in such studies have demonstrated that a rapid calcium transient occurs in immediate response to the electrical stimulus. Peak concentration of free calcium is reached within 50 msec after the stimulus in the toad sartorius muscle at 12° C (Jobsis and O'Connor 1966) and the calcium concentration is again decreased to the original level by the time of the isometric peak twitch tension. It is not possible to draw any definite conclusions from these observations as to the kinetics of calcium at the active sites in the contractile system. However, the results support the assumption (c) above) that a burst of calcium release occurs prior to and during the development of the active state.

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References

ASHLEY, C. C. and E. B. RIDGWAY: Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. *Nature* (Lond.) 1968. *219*. 1168—1169.
BRADY, A. J., Onset of contractility in cardiac muscle. *J. Physiol.* (Lond.) 1966. *184*. 560—580.

CLOSE, R, The pattern of activation in the sartorius muscle of the frog *J gen Physiol* 1962 *46* 1—18
EDMAN, K A P, The relation between sarcomere length and active tension in isolated semitendinosus fibres of the frog *J Physiol* (Lond) 1966 *183* 407—417
EDMAN, K A P and A KIESSLING, The time course of the active state of the frog skeletal muscle in relation to sarcomere length *Acta physiol scand* 1966 *68* Suppl 277 41
EDMAN, K A P and E NILSSON, The mechanical parameters of myocardial contraction studied at a constant length of the contractile element *Acta physiol scand* 1968 *72* 205—219
EDMAN, K A P, D W GRIEVE and E NILSSON Studies of the excitation-contraction mechanism in the skeletal muscle and the myocardium *Pflügers Arch ges Physiol* 1966 *290* 320—334
HILL, A V, The diffusion of oxygen and lactic acid through tissues *Proc roy Soc B* 1929 *104* 39—96
HILL A V, The heat of shortening and the dynamic constants of muscle *Proc roy Soc B* 1938 *126* 136—195
HILL, A V, The abrupt transition from rest to activity in muscle *Proc roy Soc B* 1949 *136* 399—420
JEWELL, B R and D R WILKIE, An analysis of the mechanical components in frog's striated muscle *J Physiol* (Lond) 1958 *143* 515—540
JEWELL, B R and D R WILKIE, The mechanical properties of relaxing muscle *J Physiol* (Lond) 1960 *152* 30—47
JOBSIS, F F and M J O CONNOR, Calcium release and reabsorption in the sartorius muscle of the toad *Biochem biophys Res Commun* 1966 *25* 246—252
RITCHIE, J M, The effect of nitrate on the active state of muscle *J Physiol* (Lond) 1954 *126* 155—168
SANDOW, A, Excitation-contraction coupling in skeletal muscle *Pharmacol Rev* 1965 *17* 265—320
TAYLOR, C P S, Isometric muscle contraction and the active state An analog computer study *Biophys J* 1969 *9* 759—780
WANG J H, Tracer diffusion in liquids IV Self diffusion of calcium ion and chloride ion in aqueous calcium chloride solutions *J Amer chem Soc* 1953 *75* 1769—1772
WILKIE D R The mechanical properties of muscle *Brit med Bull* 1956 *12* 177—182
WILKIE, D R, Muscle *Ann Rev Physiol* 1966 *28* 17—38

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Studies on Kinin Formation in Functional Vasodilatation of the Submandibular Salivary Gland in Cats

By

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Abstract

GAUTVIK, K *Studies on kinin formation in functional vasodilatation of the submandibular salivary gland in cats* Acta physiol scand 1970 79 174—187

The submandibular salivary gland in cats has been perfused under conditions of constant volume inflow with red blood cells suspended in normal cat plasma Such a method is favourable for studying the time course of changes in glandular vascular resistance A 30 sec supramaximal stimulation of the chordo-lingual nerve caused the vascular resistance to fall markedly within 1—6 sec When the nerve stimulation was finished a further decrease in perfusion pressure was usually observed whereafter a slow increase in vascular resistance took place

The rapidity with which the vascular response occurred indicates an initial direct action of true vasodilator nerve fibres

The second phase of the vasodilatation outlasting the chorda stimulation itself is claimed be mediated by kinin formation in the activated gland

a) When the gland was perfused with normal cat plasma so that resting perfusion pressure was low (under 55 mm Hg) then the vasodilator response to chorda stimulation changed developing gradually and outlasting the nerve stimulation

b) Reduction of kininogen 2 in the plasma perfusate resulted then in a reduced vasodilator response to chorda stimulation

c) With no kininogen 2 in the cell free perfusate stimulation of the chordo-lingual nerve caused only a very small vasodilatation or no vasodilatation at all

d) Addition of partly purified kininogen 2 to such perfusate did then restore the chorda induced vasodilatation

The development of functional vasodilatation in glandular organs has been much explored by physiologists For technical reasons most of the work in this field has been performed on salivary glands Many experimental procedures can be conveniently carried out on these organs since they are superficially located and since their functional nerve and vascular supplies can easily be isolated

There is still no general agreement as to the existence of true functional hyperaemia in glands or how this eventually is brought about A century ago Claude Bernard (1858) demonstrated that stimulation of the chorda tympani nerve produced vasodilatation in the submandibular gland and Heidenhain (1872) showed that this effect was not abolished by atropine in doses which blocked secretion of saliva As a result mainly of these findings it became accepted that the hyperaemia in the gland

was brought about through the effect of separate vasodilator nerve fibres to its vascular bed

In 1914 Barcroft challenged the view that the chorda tympani did supply the submandibular gland with separate secretory and vasodilator nerve fibres He showed that the chorda induced vasodilatation was accompanied by an increase in oxygen consumption even in atropinized non secreting salivary glands Barcroft thought that the chorda mediated vasodilatation was secondary to the increased metabolic activity in the gland *i e* a true functional vasodilatation although he did not discard the possibility that under normal circumstances dilatation may be instituted by dilator fibres and maintained by metabolic products

In 1936 Werle and Roden found that a potent vasodilator substance kallikrein was present in salivary glands and they showed that its hypotensive effects were atropine resistant In the same year Ungar and Parrot suggested that this substance was the chemical mediator of the chorda tympani provoked vasodilatation in the submandibular salivary gland The experiments performed by Hilton and Lewis (1955 a b 1956) indicated that kallikrein played a part in this vasodilatation They perfused the cat salivary gland with oxygenated Locke s solution and were able to demonstrate the presence in the effluent perfusate of a stable vasodilator material which formed bradykinin when incubated with a pseudo-globulin preparation

In recent years the view that release of kinin forming enzyme with a subsequent kinin formation is the cause of functional vasodilatation in salivary glands has been strongly opposed by several authors (Bhoola *et al* 1965 Schachter and Beilenson 1967 Skinner and Webster 1968 Schachter and Beilenson 1968) They interpret their results in the light of the old view that true vasodilator nerve fibres mediate the vasodilatation induced in the submandibular salivary gland on chorda tympani stimulation They maintain that formation of kinins plays no significant role at all in this connection

There is morphological evidence (Garrett 1966 a b c d) for an extensive innervation of the arteries and arterioles of the cat submandibular gland with cholinesterase positive nerves Denervation studies offered strong evidence that the muscular blood vessels within the parotid gland receive a dual parasympathetic and sympathetic innervation In the case of the submandibular gland the evidence is less complete because of the difficulties in performing post ganglionic parasympathectomy However after sympathetic denervation only a small reduction in the number of nerves associated with muscular blood vessels was seen

The present investigation represents an attempt to analyse the mechanism(s) behind the vasodilatation which is seen in the submandibular salivary gland of the cat on chorda stimulation The intention was to assess the significance of kinin formation for the development of the chorda mediated hyperaemia and to test whether any direct effect of vasodilator nerves could be demonstrated

The evidence given is interpreted in favour of such an effect being present and also of kinin formation playing a role in the vasodilatation mediated by the chorda tympani nerve

Methods and materials

Animals and surgical procedures Cats (2—3 1/2 kg) of both sexes were anesthetized with intraperitoneal injections of 30—40 mg/kg of pentobarbitone (Nembutal®, Abbott Laboratories London) The experiments were carried out on their perfused submandibular salivary gland A midline incision was made from the mandible to the midtracheal level After insertion of a tracheostomy tube the arterial inflow to and the venous outflow from the gland were completely isolated All branches from the common carotid artery except the one to the gland and the lingual artery were tied off Polyethylene tubing was inserted retrogradely into the lingual artery, and the gland could then be perfused through this catheter with a peristaltic [illegible] ns joining the external [illegible] The external jugular [illegible] gh polyethylene tubing [illegible] er via a preamplifier From the drop counter the blood was returned to the animal via polyethylene tubing in the ipsilateral external jugular vein

Perfusion arrangement The details of the perfusion technique are shown in Fig 1 During each perfusion period the common carotid artery was clamped and constant inflow of perfusate established at a flow rate which equalled that of the resting gland The perfusion pressure was recorded with a Statham pressure transducer (P 23 De) which was connected via a 232 DC carrier preamplifier to a multichannel recorder (Electro-Medical Engineering Co Burbank, Calif) *A change in the resistance of the vascular bed in the gland was thus reflected as a change in the perfusion pressure*

In experiments where resuspended red blood cells were used these were oxygenated by exposure to air while falling from a reservoir and down the sides of a wire mesh arrangement before entering the peristaltic pump (Fig 1) At the outflow side of the pump the red blood cells were mixed with a non-corpuscular solution which passed through a second pump channel (Fig 1) The haematocrit of this final mixed perfusate varied from 8 to 35 per cent

Injections into the arterial blood or perfusion fluid were made through a T piece connected to the polyethylene tubing which cannulated the lingual artery This T piece was placed close to the entry of the catheter into the artery

The rectal temperature of the animal was kept at 37° C The operation wound was covered with warm wet compresses and the temperature in the field of the salivary gland was about 35° C

Intravenous injections were made through a cannula in the femoral vein *Femoral arterial blood pressure* was recorded with a Statham pressure transducer (P23 De)

Heparin (Novo) 500 I U /kg was injected i v before carrying out the various cannulations A repeated injection of heparin was carried out every second hr

Various perfusates used were

1 *Heparinized cat plasma* was prepared from blood obtained through a cannula inserted into a femoral artery The blood was centrifuged at 1500 × g for 30 min Blood was collected and plasma prepared in siliconized equipment In this paper the designation kininogen 2 is used synonymously with substrate 2 which is the substrate for glandular kallikrein

2 *Kininogen 2 depleted plasma* was prepared by incubating plasma with cat saliva (4 1) for 20 hrs at 37° C the mixture being tested for the presence of kininogen 2 as previously described (Gautvik 1969)

3 *Modified Krebs Ringer solution* with 3—4 % w/v of bovine albumin (Sigma fraction 5 No A 4503) This solution contained (in meq/l) Na 147 1 K 5 9 Ca^{2} 5 0 Mg^{2} 2 4 Cl 128 4 SO_4^{2} 2 4 HCO_3 24 7 P as phosphate 36 9 mg/l

4 *Horse serum* was obtained by coagulation of whole horse blood and centrifugation at 1500 × g for 30 min Before being used it was adsorbed with red blood cells from the cat in a way which is described in greater detail elsewhere (Gautvik 1970b)

5 *A suspension of red cells* was obtained from heparinized cat blood by 3 centrifugations at [illegible]

6 *A concentrated solution of partly purified kininogen 2* was prepared as described [illegible] mixed with the kininogen free perfusates

Kininogen free perfusates or perfusates with a low kininogen content were usually obtained by mixing two of the perfusates 1 2 3 or 4 so that the concentration of kininogen 2 was less than 200 ng/ml By this procedure the amount of kininogen 1 which is normally only 1/5 of

the total kininogen present in cat plasma (Gautvik 1969), was reduced to negligible amounts Before passing the pump the perfusates were heated to 37° C All noncorpuscular perfusates ... (Millipore Filter Corporation, Bedford, Mass , U S A)

at 38° C with the Astrup Micro Equipment

1 1 with 0 9 % NaCl and used as a source of glandular kallikrein

Nerve stimulation The chordo-lingual nerve was dissected, cut, and the distal end connected to a platinum fluid electrode The indifferent electrode was placed on one leg The nerve was stimulated supramaximally (9—14 V) with square wave pulses of 1 msec duration and at frequencies of 20 per sec The duration of a period of stimulation was 30 sec unless otherwise stated

Control of the functional state of the gland and of the vascular anatomy Before the experiments were started the functional state of the gland was checked by a brief stimulation of the chordo-lingual nerve A normal gland responded to such a stimulation with lively salivation and a 5 to 20-fold increase in blood flow For evaluation of the perfusions performed at constant inflow, it was essential that the gland received no additional arterial supply This was controlled in the following way The common carotid artery was clamped and the venous outflow from the gland observed for 1 min During this period the venous outflow ceased or was very much reduced The chordo lingual nerve was then stimulated for 15 sec and the venous outflow observed continuously for another min If an increase in the venous outflow from the gland took place on chorda stimulation the gland was judged to have an extra arterial supply which had made possible the response to nerve stimulation If such an aberrant artery was not found and isolated, then that particular gland could not be used for experimental

kininogen 2, could be kept at −20° C for several weeks without losing its biological activity as tested by its ability to form kinins when incubated with the glandular kallikrein Table I gives the protein content, pH and the mean values of kininogen 2 at each step in the purification and concentration procedures

Determination of kininogen 1 and 2 The amounts of kininogen 1 and 2 in cat plasma and in other kininogen-containing solutions were estimated in terms of bradykinin equivalents that could be formed per ml (Gautvik 1969), using the isolated rat uterus preparation for evaluation of kinin activity

Results

The experimental situation described in Methods and Fig 1 is favourable for studying the time course of changes in vascular resistance in the gland During all perfusion periods the common carotid artery was clamped and the gland received a constant perfusate inflow equal to its blood supply in the normal, resting state Since the arterial inflow to the gland was kept constant, changes in vascular hinderance were immediately reflected by alterations in perfusion pressure The perfusion periods were standardized, each lasting 10 min, and supramaximal chorda stimula-

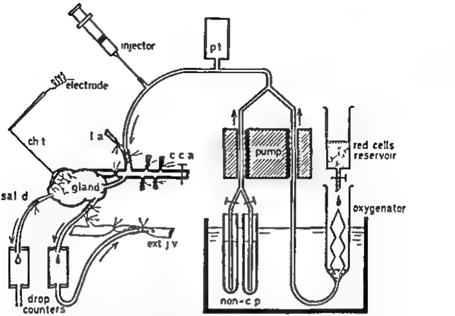


Fig 1 Schematical drawing of preparation of the cat submandibular salivary gland with its perfusion arrangement Arterial and venous vessels to the gland have been completely isolated All branches from the common carotid artery (c c a) have been ligated except the one to the gland and the lingual artery (l a.)

Perfusion pressure was recorded by a pressure transducer (p t) For perfusion using red cells [illegible]

The chordo lingual nerve (ch t) was dissected free, cut and its distal end connected to a stimulation electrode

tion for 30 sec was carried out 6—7 min after beginning the perfusion period (see Methods) In between the perfusion periods the common carotid artery was unclamped and the natural blood supply to the gland reestablished The type of vasodilatator response seen on chordo-lingual stimulation turned out to depend on the *level of the perfusion pressure existing at start of stimulation* This pressure level, which reflects the resistance to flow in the vascular bed of the gland, depends much on whether a cell-free or a red blood cell-containing perfusate was used

Experiments with perfusates containing red blood cells When washed red blood cells, resuspended in normal heparinized cat plasma, were used as perfusate, relatively high perfusion pressures prevailed The perfusion pressure stabilized usually fairly quickly at levels varying from 55—160 mm Hg In the left part of Fig [illegible] is shown how the blood flow increased in a gland with its normal circulation intact as a result of a 30 sec chorda stimulation To the right is illustrated the effect of the same type of nerve stimulus applied to the same gland now perfused at a constant flow rate as described The vasodilator response is here recorded as a decrease in per-

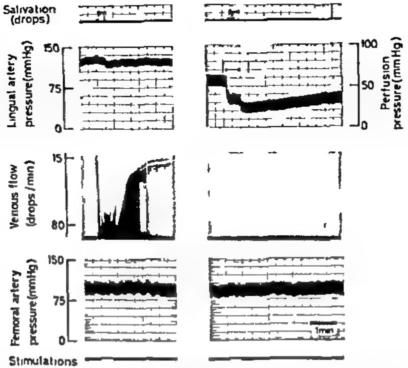
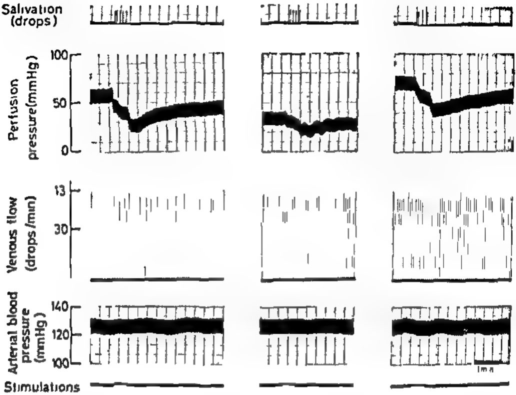


Fig 2 Effects on vascular resistance in the submandibular salivary gland of supramaxima stimulation of the chordo-lingual nerve In the left panel is shown the results of stimulatio carried out during a period of normal arterial blood supply to the gland The right panel show the effects of nerve stimulation in the same gland perfused at constant volume inflow with re cells suspended in normal plasma (haematocrit 28 per cent) Femoral artery pressure is show in both situations Stimulation of the chordo-lingual nerve carried out for 30 sec at signals

fusion pressure The secretory responses to the stimulations as well as arterial bloo pressure are also shown

The pattern of chorda induced vasodilatation presented to the right in Fig 2 wa a constant finding for glands perfused with resuspended red blood cells in norma cat plasma A two-phasic form of the response was recognized in 28 out of 30 experi ments The first phase which came on within 1—6 sec was represented by an initial fast drop in vascular resistance which levelled out before nerve stimulation ended After chorda stimulation was finished the maximal decrease in perfusion pressur usually developed as a second phase After stimulation the perfusion pressure returne slowly to prestimulation value within 6—10 min, or stabilized at a somewhat lowe level Such a long lasting vasodilatation was seen in all glands perfused with red cell suspended in normal plasma This pattern of vascular response to chorda stimula tion could be the result of one fast acting mechanism and another more graduall developing but more long lasting one

As would be expected the size of the vasodilator response to chorda stimulatio varied with the level of perfusion pressure being smaller at low than at high pres-



ig 3 Effects of chordo-lingual nerve stimulation on circulation in submandibular salivary d perfused at different haematocrits Constant volume inflow perfusion with red cells nded in normal cat plasma In the three perfusion periods shown the haematocrit was m the left to right 28 8 and 28 per cent respectively Supramaximal stimulation of the ordo-lingual nerve for 30 sec at signals Between the perfusion periods normal circulation vas restored for 10 min

sures With a perfusion pressure of 50 mm Hg or less the vasodilator pattern was also different from that seen during higher pressures The slope of the initial pressure drop became less steep and the main part of the vasodilator response often developed after the nerve stimulation was finished The long-lasting character of the vasodilator response did persist however, at such low perfusion pressures as long as the perfusate contained kininogen 2 During experiments performed at low perfusion pressure, there was apparently conditions in the gland allowing the mechanism responsible for the more slowly developing and more long-lasting vasodilator mechanism to prevail This mechanism could thus perhaps be studied more separately during low pressure conditions In Fig 3 is shown an experiment in which the same gland is perfused at equal flow rates but at two different perfusion pressures The perfusate was normal plasma with resuspended red blood cells In the tracing to the left and right the haematocrit was about 28 per cent in the middle only 8 per cent The vasodilator response occurring in the middle of Fig 3 could represent mainly the slowly developing mechanism with its long-lasting vasodilator effect

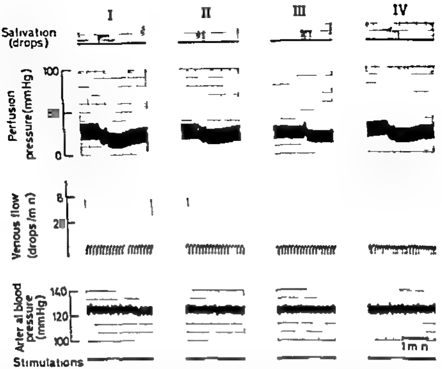


Fig 4 Effects of chordo-lingual nerve stimulation on circulation in perfused submandibular salivary gland on altering perfusate Constant volume inflow perfusion with normal plasma (I and IV) and plasma diluted 2 1 (II) and 1 1 III) with 4 per cent albumin (w/v) in Krebs-Ringer solution Supramaximal stimulation of the chordo-lingual nerve for 30 sec at signals. Between each period of perfusion normal circulation was restored as described for Fig 3

Gautvik *et al* (1970) perfused the cat submandibular salivary gland at a constant flow rate with blood from the animal's femoral artery and found that the amount of substrate for glandular kallikrein (kininogen 2) was reduced by 30 to 60 per cent in the venous effluent during and immediately after activation of the gland Since kinin formation apparently takes place under these experimental conditions it is important to know to what extent such formation was responsible for the observed vasodilator response to chorda stimulation Cat glandular kallikrein acts preferentially on the so-called kininogen 2 in plasma When a perfusate without substrate for glandular kallikrein is passed through the gland kinin formation can no longer take place if the interstitial store of kininogen is depleted

Experiments performed with non-corpuscular perfusates A more detailed study of the relationship between the composition of the perfusate and the mechanism(s) responsible for the long lasting vasodilator effect of chordo-lingual stimulation was therefore carried out Non-corpuscular perfusates were used in order to obtain uniform and low perfusion pressures The effect of diluting normal plasma with a 4 % albumin Krebs-Ringer solution was first examined in 2 cats To the left and right in

TABLE I Purification and concentration of kininogen 2 from cat plasmas The purification was carried out according to the method of Jacobsen (1966) The kininogen containing solutions were concentrated as described by Flodin *et al* (1960) The figures in the Table represent mean values from III different cat plasmas which were individually treated

	Volume (ml)	Content of protein (mg/ml)	Content of kininogen 2 μg/ml)	pH	Yield of kininogen 2 (%)
Plasma	25	64 6	1 6	7 65	
After gel filtration on Sephadex G 200	40	13 6	0 76	7 9	76
After dialysis	40	13 6	0 76	7 67	100
After concentration with Sephadex G 25 coarse, 1st time	16 2	33 5	1 85		98
After concentration with Sephadex G 25 coarse 2nd time	6 4	84 6	4 68	7 8	100

Fig 4 (period I and IV) are shown the effects of a 30 sec supramaximal chorda lation when the gland was perfused with normal cat plasma During the tests of ods II and III the gland was perfused with normal plasma that had been diluted and 1 1 with albumin Krebs Ringer solution respectively The normal vasotor effects of chorda stimulation were considerably and progressively reduced as a result of the perfusate dilution The same results were seen when normal plasma diluted with kininogen depleted plasma was used as perfusate The vasodilatation induced on chorda stimulation at low perfusion pressures could no longer be demonstrated if the amount of kininogen 2 in the perfusion fluid was less than 400—500 ng/ml In 8 cats kininogen free solutions were used and no vasodilator response was then seen on chorda stimulation at low perfusion pressures while control perfusions with normal plasma elicited a vasodilatation Under such conditions where the vasodilator effect of chorda stimulation was very much reduced or abolished close arterial injections of 1 μg synthetic bradykinin still gave a good vasodilator response

As a next step an answer to the following question was sought Could the addition of kininogen 2 in a partly purified form to a perfusate which lacked this kininogen restore the vasodilatation evoked by chorda stimulation? Experiments were undertaken in 4 cats in order to analyse this problem In Fig 5 are shown the results from one of these experiments In perfusion periods I and V is seen the usual chorda induced vasodilator response with normal plasma as perfusate Periods II and IV show the effect of nerve stimulation when the gland was perfused with a kininogen free solution In period III the gland is perfused with the same kininogen free solution in which had been added partly purified kininogen 2 With the kininogen free

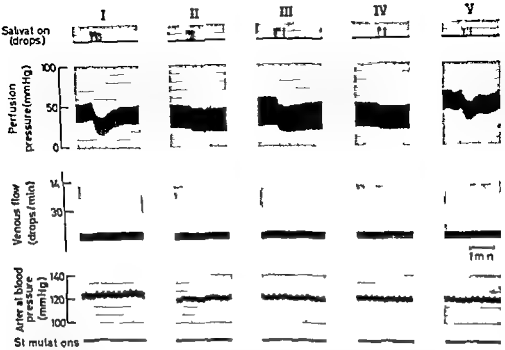


Fig 5 Effects of chordo-lingual nerve stimulation on circulation in submandibular salivary gland perfused with various non-corpuscular perfusates Five successive constant volume inflow perfusions are shown During perfusions I and V normal cat plasma was used as perfusate in perfusions II and IV kininogen free solution was used and in III partly purified kininogen 2 was added to the kininogen free solution Stimulation of chordo-lingual nerve for 30 sec at signals. Each perfusion lasted 10 min and normal circulation was restored in between as described for Fig 3

perfusates the vasodilator effect of chorda stimulation was abolished or hardly detectable A good vasodilator response could however be demonstrated after addition of kininogen 2 to these perfusates Kininogen 2 was added as a concentrated solution which contained 4—7 μg/ml This gave a final kininogen concentration in the perfusate of 1—1 5 μg/ml which is comparable to the physiological values in cat plasma (Gautvik 1969) Without exception such addition of kininogen 2 to kininogen free perfusates almost restored the chorda mediated vasodilatation at low perfusion pressures The results from experiments on 4 cats are summarized in Table II

Alterations in P_{O_2} Because of the constant inflow type of perfusion the oxygen supply to the gland could be grossly deficient during periods of chorda stimulation. The P_O of the control perfusates and of the test perfusates was equal so that hypoxic effect should be the same throughout one experiment with different perfusates Still marked hypoxia might interfere with the vasodilator responses developing during chorda stimulation It became important therefore to know the possible effect of an accompanying hypoxia during chorda stimulation Perfusions were carried out with red cells resuspended in normal plasma and with P_O of the perfusion fluid a

TABLE II Vasodilator effect of chorda-lingual nerve stimulation was recorded as a decrease in perfusion pressure in per cent of initial pressure level the glands being perfused at a constant flow rate Non corpuscular solutions were used as perfusates (for details see Methods) The results from experiments in 4 cats are presented More than one series of perfusions were usually performed in each cat The mean values and ranges are given for each animal, and also the total number of tests for each type of perfusate

	Cat no	Perfusion with			
		Normal plasma	Kininogen 2 depleted solution	Kininogen 2 restored solution	Normal plasma
Maximal decrease in perfusion pressure after chorda-lingual stimulation (%)	1	52	1	21	52
	2	14 (11—16)	0	15	18 (12—23)
	3	31 (25—36)	6 (5—7)	17 (8—24)	10 (10—10)
	4	18 (15—23)	0	15 (15—15)	15
No of total chorda-lingual stimulations		9	7	9	6

600 mm Hg 160 mm Hg and 5 mm Hg respectively The equilibrating gas mixtures contained 5 % CO_2 The variation in P_{O_2} of the solutions, which at times rendered the gland extremely hypoxic did not change the normal chorda induced vasodilator pattern When a perfusate having a P_{O_2} of 5 mm Hg was used this did in itself cause a decrease in the vascular resistance but it did not however alter the vasodilator pattern caused by chorda stimulation Similarly isolated reductions in P_{CO_2} content of the perfusate caused no change in the vascular response to chorda stimulation

Discussion

Findings presented here indicate the presence of two vasodilator mechanisms in the submandibular salivary gland of cats The onset of chorda induced vasodilatation was very quick with a latency of 1—6 sec This was well seen when perfusates containing red blood cells were used giving a relatively high perfusion pressure It seems unlikely that a mechanism involving release of an enzyme from gland cells and its successive participation in chemical reaction(s) can be that effective within a few sec. When ellagic acid a compound which when present in plasma will result in kinin formation was injected intravenously into rat hamster or guinea pig a temporary hypotensive period followed The maximal hypotensive effect occurred 1/2—1 min after the injection started (Gautvik and Rugstad 1967 Gautvik 1969) The first rapid phase of the chorda induced vasodilatation was thus apparently brought about by the action of vasodilator nerve fibres

The later phase of the vasodilator response to chorda stimulation observed in these experiments however, strongly suggest formation of kinins secondary to activation of the gland This view is based on the following observations

1 When the gland was perfused at low perfusion pressures the slowly developing and long lasting vasodilatation seen on chorda stimulation depended on the presence of kininogen 2 in the perfusate A reduction in the vasodilator response to chorda stimulation could be demonstrated when the kininogen content of the perfusate plasma was reduced

2 No vasodilator effect of nerve stimulation could be demonstrated when kininogen free perfusates were used

3 *The addition of partly purified kininogen 2 to such perfusates did restore*, however, vasodilatation on chorda stimulation

The stepwise reduction in perfusion pressure observed on chorda stimulation with maximal dilatation occurring with cessation of stimulation could represent a spread of current to sympathetic nerve cells or the effect of stimulating vasoconstrictor nerve fibres running in the chordo lingual nerve The existence of such vasoconstrictor nerve fibres in chorda are, however, not generally accepted (for review see Emmelin, 1967) A possible effect of myoepithelial cell contraction induced by chorda stimulation on the perfusion pressure could not be excluded

When the gland was perfused with non-corpuscular perfusates containing no kininogen 2 (see Methods), chorda stimulation had a very small vasodilator effect or no such effect at all This lack of effect was apparent after a perfusion period of 6—8 min If kinin formation takes place essentially in the interstitial fluid on chorda stimulation such a period of perfusion did apparently deplete the gland of its extra vascular content of kininogen 2

During control experiments the gland was exposed to an isolated alteration in the P_{O_2} content of the perfusate which at times rendered the organ extremely hypoxic Such a variation of the perfusate P_{O_2} content did not change the normal chorda induced vasodilator pattern An isolated effect of glandular hypoxia during these experiments seemed thus to be of little significance for the interpretation of the results presented

Low pressures of perfusion prevailed during the experiments where non corpuscular perfusates were used Under these non physiological conditions the effect of kininogen depletion and readdition to glandular perfusate was easily demonstrated on the chorda mediated vasodilatation The important question is whether the two vasodilator mechanisms apparently operating in the present experiments are active to the same extent also during more physiological conditions It is not unreasonable to believe that an organ of this type should have a dual way of securing an adequate blood flow and blood distribution during activity Stimulation of vasodilator nerve fibres in the chordo-lingual nerve provides the gland with an initial rapid and marked increase in flow which can cope with the first demands for solutes and oxygen The simultaneous activation of the kinin forming system on chorda stimulation could assist the nerve initiated vasodilatation and make possible a maintained

increase in flow after the nerve stimulation has ended A chemical vasodilator mechanism of this type could also serve to distribute blood flow within the gland in accordance with local needs. Barcroft and Piper (1912) showed that the increased oxygen utilization in the gland initiated by chorda stimulation outlasted the salivary secretion by several min The prolonged vasodilator effect of chorda stimulation could thus serve the same mechanism in glandular organs as does local chemical changes in active muscles According to Barcroft and Piper such a mechanism 'serves to replenish a store of potential energy which is liberated in the act of secretion'

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References

BARCROFT J In *The respiratory function of the blood* 1914 1st ed pp 140—148 Cambridge University Press

BARCROFT J and H PIPER The gaseous metabolism of the submaxillary gland with reference especially to the effect of adrenalin and the time relation of the stimulus to the oxidation process. *J Physiol* (Lond) 1912 *44* 359—373

BERNARD C *De l'influence de deux ordres de nerfs qui determinent les variations de couleur du sang veineux dans les organes glandulaires* *C R Acad Sci* (Paris) 1858 *47* 245—253

BHOOLA K D J MORLEY M SCHACHTER and L H SMAJE Vasodilatation in the submaxillary gland of the cat *J Physiol* (Lond) 1965 *179* 172—184

EMMELIN N Nervous control of salivary glands 1967 In *Handbook of Physiology Alimentary Canal* Volume II pp 595—615 Waverly Press Inc Baltimore Maryland 21202 U S A

FLODIN P B GELOTTE and J PORATH A method for concentrating solutes of high molecular weight *Nature* (Lond) 1960 *188* 493—494

GARRETT J R The innervation of salivary glands I Cholinesterase positive nerves in normal glands of the cat *J roy micr soc* 1966a *85* 135—148

GARRETT J R *The innervation of salivary glands II The ultrastructure of nerves in normal glands of the cat* *J roy micr Soc* 1966b 85 149—162

GARRETT J R The innervation of salivary glands III The effects of certain experimental procedures on cholinesterase positive nerves in glands of the cat *J roy micr Soc* 1966c *86* 1—13

GARRETT J R The innervation of salivary glands IV The effects of certain experimental procedures on the ultrastructure of nerves in glands of the cat *J roy micr Soc* 1966d *86* 15—31

GAUTVIK K Kininogen in plasma from cat hamster and guinea pig *Acta physiol scand* 1969 *76* 191—200

GAUTVIK K *The interaction of two different vasodilator mechanisms in the chorda tympani activated submandibular salivary gland* *Acta physiol scand* 1970b 79 188—203

GAUTVIK K M S M HILTON and S H TORRES Consumption of kininogen in the submandibular salivary gland when activated by chorda stimulation *J Physiol* (Lond 1970 (To be published

GAUTVIK K M and H E RUGSTAD Kinin formation and kininogen depletion in rats after intravenous injection of ellagic acid *Brit J Pharmacol* 1967 *31* 390—400

HEIDENHAIN R Über die Wirkung einiger Gifte auf die Nerven der glandula submaxillaris *Pflug Arch ges Physiol* 1872 *5* 309—318

HILTON S M and G P LEWIS The cause of the vasodilatation accompanying activity in the submandibular salivary gland *J Physiol* (Lond) 1955a *128* 235—248

HILTON S M and G P LEWIS The mechanism of the functional hyperaemia in the submandibular salivary gland *J Physiol* (Lond) 1955b *129* 253—271

HILTON S M and G P LEWIS The relationship between glandular activity, bradykinin formation and functional vasodilatation in the submandibular salivary gland *J Physiol* (Lond) 1956 *134* 471—483

JACOBSEN [illegible] Separation of two different substrates for plasma kinin forming enzymes Nature (Lond) 1966 *210* 98—99
KALCKAR … of purine compounds by means of specific enzym
SCHACHT … nd vasodilatation in the submaxillary gland *Gastro*
SCHACHTER M and [illegible] BEILENSON Mediator of vasodilatation in the submaxillary gland *Fed Proc* 1968 27 73—75
SKINNER N S and M E WEBSTER Kinins beta adrenergic receptors and functional vasodilatation in the submaxillary gland of the cat *J Physiol* (Lond) 1968 *195* 505—519
UNGAR G and J L. PARROT Sur la presence de la callicreine dans la salive et la possibilite de son intervention dans la transmission chimique de l influx nerveux *C R Soc Biol* (Paris) 1936 *122* 1052—1055
WERLE E and P VON RODEN Über das Vorkommen von Kallikrein in den Speicheldrüsen und im Mundspeichel *Biochem Z* 1936 *286* 213—219

Acta physiol scand. 1970 79. 188—203

From the Institute of Physiology, University of Oslo, Norway

The Interaction of Two Different Vasodilator Mechanisms in the Chorda-Tympani Activated Submandibular Salivary Gland

By

Kaare Gautvik

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Abstract

Gautvik K *The interaction of two different vasodilator mechanisms in the chorda tympani activated submandibular salivary gland* Acta physiol scand 1970 79 188—203

The vasodilatation caused by stimulation of the chordo lingual nerve has been studied in the cat submandibular salivary gland perfused under conditions of constant volume inflow, at a rate equ...

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...ulations were carried out during a single perfusion period When the gland was perfused continuously with red cells suspended in a kininogen free solution then one of two different response patterns could be seen When a low perfusion pressure prevailed (below 55 mm Hg), then the chorda mediated vasodilatation was reduced or abolished on repeated nerve stimulation At conditions with a higher perfusion pressure (60—120 mm Hg) there developed on chorda stimulation a one phasic short lasting vasodilatation which was not reduced on repeated stimulations

In another series of experiments the kinin destroying activity of a perfusate of red cells in normal plasma was increased by addition of carboxypeptidase B Chorda stimulation then caused a short lasting vasodilatation of the type seen when a red cell perfusate containing no kininogen was used

It is concluded from these findings that functional vasodilatation in the submandibular salivary gland is probably initiated by the effect of vasodilator nerve fibres and supported and maintained by the action of kinins formed in the activated gland

The results of previous studies (Gautvik *et al* 1970 Gautvik 1970 a) indicate that both kinin formation and dilator nerve fibres play a role in the vasodilatation which develops in the submandibular salivary gland of the cat on stimulation of the chordo-lingual nerve An initial vasodilator phase appeared to be mediated through the direct action of vasodilator nerve fibres whereas the action of kinins formed was subsequently added to this initial effect (Gautvik 1970 a)

The present investigation was carried out in order to evaluate in more detail the two vasodilator mechanisms and their interaction in the gland Some of the most

important arguments which have been advanced against the existence of a chemically mediated vasodilator mechanism in salivary glands were also examined Of particular importance in this respect is the claim of Bhoola *et al* (1965) that stimulation of the chordo lingual nerve caused vasodilatation in a submandibular salivary gland perfused with horse serum a perfusate which did not develop kinins when incubated with cat glandular kallikrein Skinner and Webster (1968) also brought forward evidence which they considered to be at variance with the theory that kinin formation is involved in the chorda mediated vasodilatation of salivary glands They found that intravenous injection of carboxypeptidase-B a potent kinin inactivating enzyme, abolished the dilator effect of close arterial infusions of bradykinin, whereas there was apparently no effect on the chorda induced vasodilatation in the submandibular salivary gland

Methods and materials

Surgical procedures and perfusion arrangement The experiments were carried out on the perfused submandibular salivary gland of cats (2—4 kg) which had been anesthetized with i p injections of 30—40 mg/kg of pentobarbitone (Nembutal®, Abbott Laboratories London) After cannulating the trachea, the arterial inflow to and the venous outflow from the gland

recorded by a Statham pressure transducer (P 23 De) connected to a multitrace recorder (Electro-Medical Engineering Co Burbank Calif) equipped with a 232 DC carrier pre

lateral jugular vein

Red blood cells used in the perfusion experiments were *oxygenated* as described by Gautvik (1970 a) The haematocrit of the final mixed perfusates entering the gland varied from 20 to 35 per cent,

Arterial injections or *injections into the perfusion* fluid were made through a T piece in the polyethylene tubing cannulating the lingual artery close to the gland

Intravenous injections were made through a cannula in the femoral vein Femoral arterial blood pressure was recorded by a Statham transducer (P 23 D+) *Heparin* (Novo) 500 I U / kg was injected i v before cannulations were carried out Repeated injections of heparin (500 I U /kg were carried out every second hour

Perfusates used

1 *Heparinized cat plasma* was prepared from blood obtained through a cannula inserted into the femoral artery of a donor cat The blood was centrifuged at $500 \times g$ for 30 min. Siliconized equipment was used.

In this investigation the term kininogen 2 is used synonymously with the term substrate 2 as the substrate for glandular kallikrein

2 *Cat plasma depleted of kininogen* 2 and

3 *Modified Krebs Ringer solution with 4 per cent (w/v) bovine albumin* was prepared as described by Gautvik (1970 a)

4 *Horse serum* was obtained after coagulation of whole horse blood and after centrifugation at $1500 \times g$ for 30 min Before being used as a kininogen free perfusate the serum was adsorbed with cat red cells as described below

5 *Suspensions of red cells* A nearly pure stock suspension of red cells was obtained from heparinized cat blood as described by Gautvik 1970 a) Perfusates containing red cells were prepared from the red cell stock suspension by addition of perfusates 1 2 3 4 or mixtures of these to the desired haematocrit

B *A concentrated solution of partly purified kininogen 2* was prepared as previously described (Gautvik 1970 a)

Kininogen-free perfusates or perfusates with a low kininogen content were usually obtained by

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Removal of naturally occurring horse serum agglutinins against cat red cells Horse serum was obtained as described and diluted progressively (1/1—1/64) with 0 9 % NaCl and tested against a 2 % (v/v) suspension of washed cat red cells originating from 3 different cats Two parts of diluted serum and one part of the suspended red cells were incubated for 1 hr at 37° C,

depleted of cat red cell agglutinins

Control procedures for evaluation of the functional state of the gland and of its vascular anatomy Before perfusion experiments were started the functional state of the gland was checked by a brief stimulation of the chordo-lingual nerve A normal gland responded to such stimulation with marked salivation and a 5—10 fold increase in blood flow For the evaluation of perfusion experiments performed at constant flow rate, it was essential that the gland should receive no additional arterial blood supply The previously described procedure for testing the arterial inflow to the gland (Gautvik 1970 a) was followed

Salivary secretion The submandibular ducts were exposed in the floor of the mouth and cannulated with polyethylene tubing distal to the place where the chordo-lingual nerve crosses the ducts The flow of saliva was registered with a drop counter The collected saliva was diluted 1 1 with 0 9 % NaCl and used as a source of glandular kallikrein

Nerve stimulation The chordo-lingual nerve was freed and cut, and the distal end connected o a platinum fluid electrode The indifferent electrode was placed on one leg The nerve was timulated supramaximally (9—14 V) with square wave pulses at 1 msec duration and at frequencies of 20 per sec The duration of a period of stimulation was 30 sec

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was added to 4 9—4 8 ml of normal cat plasma. The enzyme concentrations of carboxypeptidase-B in the perfusion fluids as well as in venous blood outflow and in gland homogenates, were estimated in terms of kinin-destroying activity as described below As an additional measure of carboxypeptidase B activity the ability to hydrolyze hippuryl L-arginine was also evaluated, as described by Folk *et al* (1960) As recommended by these authors the units of activity were given as per cent hydrolysis taking place per min at a substrate concentration of 0 001 M and under certain standardized conditions The ability of normal cat plasma to

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sample and that caused by known amounts of synthetic bradykinin One unit of kininase was defined as the amount of enzyme which destroyed 75 % of the 500 ng of bradykinin in 11 min under standardized conditions (Rugstad 1966, Gautvik 1969 The kininase activity of normal cat plasma varied from 1 to 20 units Gautvik 1969)

Determination of kininogen 1 and 2 The amounts of kininogen 1 and 2 in cat plasma and in other kininogen containing solutions were estimated in terms of bradykinin equivalents that could be formed per ml (Gautvik 1969), using the isolated rat uterus preparation for evaluation of kinin activity

via a tubing inserted in the ipsilateral femoral vein The nerve to the lateral head of the gastrocnemius was isolated and cut about 5 mm proximal to where it entered the muscle, and the distal end stimulated via a platinum fluid electrode Muscle contractions were induced by stimulating the nerve with square pulses at 5 V, of 1 msec duration and at a frequency of 5 per sec for 10 sec periods Alternatively stimulations at 5 V, 0 1 msec pulse duration and at a frequency of 50 per sec for 5 sec periods were used

Dialysis was carried out with Visking dialysis tubing 18—32 (Visking Department, Union Carbide International W, New York U S.A)

Drugs Acetylcholine was used as chloride (S A F Hoffman—La Roche & Co, Basel) and isoprenaline as sulphate, puriss, (Siegfried Zoingen, Switzerland) Synthetic bradykinin (BRS 640 Sandoz Basel Switzerland) was used

Results

Vasodilator responses in the cat submandibular salivary gland caused by repeated stimulations of the chordo lingual nerve

When the submandibular salivary gland was perfused at constant volume inflow with red blood cells suspended in normal cat plasma at perfusion pressures of 55—160 mm Hg, a typical vasodilator pattern was seen on chordo-lingual nerve stimulation (Gautvik 1970 a) This response, which is shown in Fig 1 was composed of an initial rapid decrease in vascular resistance inside the gland, and of a further drop in resistance which came on when the nerve stimulation ended and which outlasted the nerve stimulation by several min In all of 3 cats tested this pattern of the response was maintained on repeated stimulation of the chordo lingual nerve In Fig 1 are shown the vasodilator response to repeated supramaximal chorda stimulations for 30 sec in one of these experiments The perfusion pressure sometimes failed to return to prestimulation level, and the absolute decrease in vascular resistance would then become smaller on subsequent nerve stimulation The response pattern did not change, however The duration of the vasodilator effect of chorda stimulation was found to depend on the amount of kininogen 2 present in the perfusate By appropriate control of this amount the vascular resistance could be made to return to near initial value within a 5 min period after stimulation This was achieved by dilution of normal cat plasma with a 4 per cent (w/v) albumin Krebs Ringer solution to a final kininogen 2 concentration of 0 6 μg pr ml Such a content is within the lower normal range for cat plasma (Gautvik 1969)

When the gland was perfused with red cells in kininogen free perfusate the pattern and development of the vasodilator responses to repeated chorda stimulation depended on the *prestimulation level of the perfusion pressure* In perfusion performed at low pressures, from 30 to 55 mm Hg which was achieved by using a reduced haema-

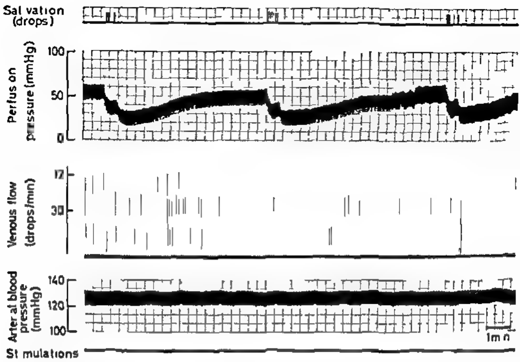


Fig. 1. Vasodilator responses in perfused cat submandibular salivary gland to repeated stimulations of the chorda-lingual nerve. The gland was perfused at constant volume inflow with red cells suspended in normal but diluted cat plasma. Haematocrit 26 per cent. The plasma had previously been diluted with a 4 per cent (w/v) albumin Krebs Ringer solution whereby kininogen 2 concentration was lowered to 0.6 $\mu g/ml$ (see the text). Stimulations of the chorda-lingual nerve for 30 sec periods at signals. For details of perfusion procedure see Methods.

tocrit the vasodilator response to repeated chorda stimulations became successively smaller or disappeared as seen to the left in Fig. 2. When at the same flow rate the perfusion pressure was increased to 90 mm Hg by increasing the haematocrit chorda stimulation still caused the initial rapid reduction in vascular resistance but recovery of vascular tone to prestimulation level starting as soon as stimulation ended was seen in all 6 cats tested. To the right in Fig. 2 is presented such a pattern of short lasting chorda mediated vasodilatation. The short lasting rapidly occurring vasodilator response was reproducible on repeated chorda stimulation. The response did not even diminish when such repeated nerve stimulations were carried out for 1 hr.

Alternative perfusions of the submandibular gland with red cells suspended in kininogen containing or kininogen free solutions

In 6 cats the submandibular gland was perfused intermittently with red cells re-suspended in normal plasma or in kininogen free solutions. Haematocrit varied from 25—30 per cent between the experiments resulting in a perfusion pressure of 50—

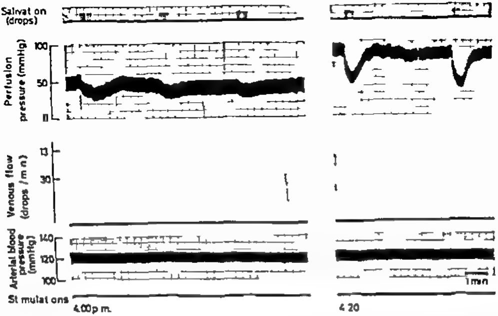


Fig. ■ Vasodilator responses to chordo-lingual nerve stimulation in cat submandibular gland perfused at a constant volume inflow with red cells suspended in kininogen free solution. The effects of repeated stimulations of the chordo-lingual nerve tested at two levels of perfusion pressure. In the left part of the figure the perfusate had a haematocrit of 20 per cent. In the period between the two parts of the figure the perfusion pressure was elevated as a result of an induced increase in haematocrit up to 33 per cent. Periods of nerve stimulations lasting 30 sec at signals.

110 mm Hg. In Fig. 3 are shown results from one of these experiments. To the left and right is shown the vasodilator response to a 30 sec supramaximal chorda stimulation with red blood cells resuspended in normal plasma as perfusate. In the middle is seen the response in the same gland to the same type of stimulation during a perfusion with red blood cells suspended in kininogen free solution. In this and 6 similar experiments the usual chorda induced vasodilator response was changed when kininogen 2 was lacking in the perfusate. The maximal fall in vascular resistance was often as marked as before, but the effect was always much more short lasting, and the perfusion pressure returned to a normal level immediately after the nerve stimulation had ended. Such a short lasting vasodilator response to chorda stimulation developed after 5—15 min of perfusion with red cells in kininogen free solution. When subsequently a perfusate consisting of red cells suspended in normal plasma was used again, the original long lasting vasodilator response could be obtained once more, but only if the animal's normal circulation had first been restored for about 1 hr (Fig. ■ right).

In 6 additional cats the submandibular salivary gland was perfused for a long continuous period with a perfusate of red cells in kininogen free solution. The pressure of perfusion varied between experiments from 70—110 mm Hg. When on

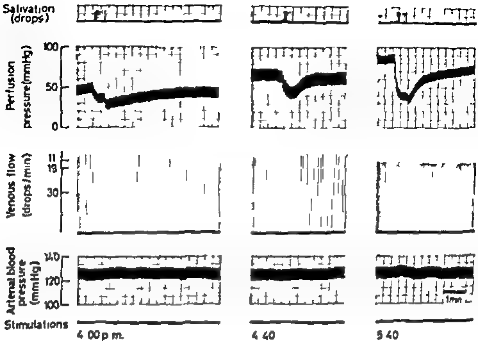


Fig 3 Effects of chordo-lingual nerve stimulation on vascular resistance in the perfused submandibular salivary gland with (on left and right) and without (middle) kininogen in perfusate Constant volume inflow perfusion with red blood cells suspended in kininogen-containing and kininogen free fluid (see text) Haematocrit was 26 per cent throughout experiment The 3 perfusion periods shown which lasted 10 min each, were separated by intervals where normal circulation was re-established through the common carotid artery For details of perfusion procedure see Methods

repeated chorda stimulation the pattern of a rapid but short lasting vasodilator response was seen kininogen 2 was added to the perfusate to a final concentration of 1—1 8 µg pr ml This level is within the limits found in normal cat plasmas (Gautvik 1969) The typical long lasting phase of the vasodilator pattern to chorda stimulation did not return however, on such an addition of partly purified kininogen II to the perfusate

Perfusion of the submandibular salivary gland with horse serum

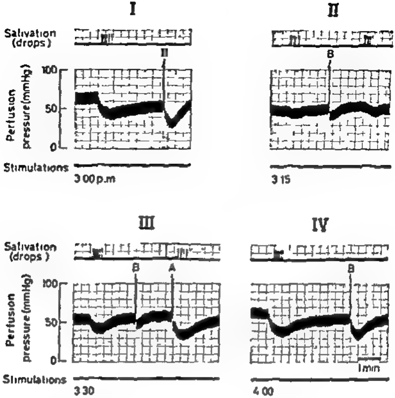
Other investigators have used horse serum as a convenient substitute for a homologous kininogen free perfusate (Bhoola *et al* 1965) Thus, when cat glandular kallikrein is incubated at 37 C with horse serum in the presence of EDTA (Aethylendiamintetraessigsäure Dinatriumsalz Dihydrat) as a kininase inhibitor, no kinins are formed When in the present work the salivary gland was perfused at constant flow rate with horse serum (see Methods) the perfusion pressure steadily increased and did not level out as was usually seen with other perfusates Clumping and aggregation of cat red blood cells could be observed in the venous polyethylene tubing The presence of agglutinating antibodies in horse serum against washed cat red cells can

be demonstrated (see Methods) The red cell agglutinins were active both at 4° C and at 37° C and they could be removed by adsorption with cat red blood cells When adsorbed horse serum was used as part of the kininogen free solutions in which red blood cells were resuspended, chorda stimulation elicited a vasodilator effect of the rapidly occurring short lasting type similar to that seen to the right in Fig 2 in all of 6 cats tested

The effect of carboxypeptidase B on the chorda lingual nerve mediated vasodilatation in the submandibular gland

In 2 different series of experiments the effect on the chorda induced vasodilatation of adding carboxypeptidase B a potent kinin inactivating enzyme to the perfusate was tested During these experiments the glands were perfused at constant flow rate for periods of 10 min and nerve stimulations were carried out after 6—7 min of perfusion. The periods of perfusion were alternated with intervals in which normal circulation through the common carotid artery was restored During perfusion of the gland with perfusates containing carboxypeptidase-B the effluent from the gland was not returned to the animal

In the first series comprising 5 expts a perfusate of red cells resuspended in normal cat plasma was used and the results obtained were uniform The pressure of perfusion ranged from 60—90 mm Hg In Fig 4 are presented typical results from one of these experiments where 4 successive perfusions were carried out as described In periods I III and IV the gland was perfused with the ordinary perfusate whereas during the second perfusion period in this sequence the same type of perfusate now containing carboxypeptidase B entered the gland Prior to the perfusion in period II 0 2 ml of the concentrated carboxypeptidase-B solution had been added to 4 8 ml of the plasma fraction of this perfusate When the gland was perfused with carboxypeptidase B a temporary decrease in perfusion pressure was always seen indicating a lowering of the vascular resistance In Fig 4 are shown the responses to 30 sec supramaximal chorda stimulations and also the effects of close arterial injections of 0 15 µg of bradykinin (B) and of 0 1 µg of acetylcholine (A) The usual vasodilator response to chorda stimulation and bradykinin injection (0 15 µg) seen in period I could no longer be observed when carboxypeptidase B was administered to the gland as in period II An increased amount of injected bradykinin 1—3 µg did however usually elicit a vasodilatation also under these conditions On the next perfusion period III when red cells in normal plasma was used as perfusate again chorda stimulation caused a short lasting vasodilatation but 0 15 µg of injected bradykinin had still no more effect than had control injections of 0 15 ml of a Krebs Ringer solution A subsequent injection of 0 1 µg of acetylcholine caused however a marked vasodilatation. At this stage the acetylcholine provoked vasodilatations were much more short lasting than when no carboxypeptidase-B had been infused into the gland (Gautvik 1970 c) During the last perfusion period IV chorda stimulation did again provoke a long lasting vasodilator response and 0 15 µg of bradykinin did now cause a decrease in perfusion pressure The vasodilator response to bradykinin injec



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suspended in normal cat plasma, and during period II the same type of perfusate now containing carboxypeptidase-B preparation (0 2 ml of concentrated enzyme solution to 4 8 ml of plasma) entered the gland Haematocrit was about 28 per cent throughout the experiment Close arterial injections of bradykinin, 0 15 μg at (B), and of acetylcholine, 0 1 μg at (A) Stimulation of the chordo-lingual nerve for 30 sec periods at signals

tions always reappeared together with the long-lasting vasodilator phase of chorda stimulation in these experiments

In the second series of experiments with carboxypeptidase-B additions, normal cat plasma was used as basic perfusate With a plasma perfusate there will prevail a low perfusion pressure which favours the analysis of the long-lasting vasodilator phase of chorda stimulation (Gautvik 1970 a) In 4 of the 5 cats tested the effect of chordolingual stimulation was abolished when carboxypeptidase-B was present in the perfusate and also in the subsequent perfusion periods with normal plasma In Fig 5 are presented results from an experiment of this type, in which the gland was perfused with normal plasma, in periods I and IV, and with plasma to which was added carboxypeptidase-B, in periods II and III During the first perfusion with the enzyme added, there was a reduction of the vasodilator response to chordo-lingual stimulation (period II). During the second perfusion with carboxypeptidase-B in the per-

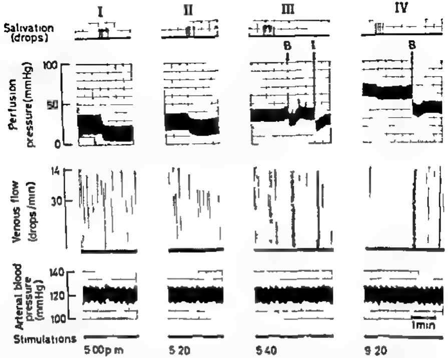

Fig 5 The effect of adding carboxypeptidase B to the perfusate on the vasodilator response to chordo-lingual nerve stimulation in the cat submandibular gland The organ was perfused with normal plasma at constant volume inflow The four (10 min) successive perfusion periods shown were interrupted by intervals of normal circulation through the ... During periods I and IV normal ... same plasma, now contai... solution to 4 9 ml of plas... (B) and of isoprenaline, ... at signals

fusate no nerve induced vasodilatation could be detected At this stage close arterial injections of bradykinin (2 μg), did cause a minute vasodilatation This is shown in Fig 5 together with the response to 4 μg of isoprenaline The vasodilator effect of chorda stimulation was only slowly and gradually restored during the subsequent perfusions with normal plasma interrupted by intervals of established circulation through the carotid artery About 4 hrs after the first introduction of carboxypeptidase-B, chorda stimulation caused a small vasodilatation and the vasodilator effect of injected bradykinin, 2 μg, was then again marked (period IV)

In Fig 6 are presented the estimates of kinin destroying activity (A) and of the hippuryl L arginine splitting activity (B) in the effluent perfusate during a typical perfusion with carboxypeptidase-B containing plasma Measurements are also given from the venous outflow immediately after the carotid artery had been reopened (arrows) The bradykinin and hippuryl L arginine splitting activity of the effluent fluid increased during the first 4—6 min of perfusion and thereafter the values

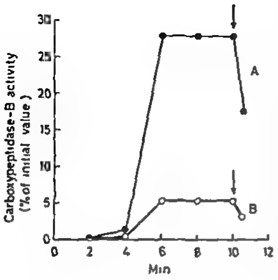

Fig 6 The concentration of carboxypeptidase B in effluent perfusate and venous blood from submandibular salivary gland during and immediately after a perfusion experiment The unclamping of the carotid artery is marked with arrows The carboxypeptidase B activity is expressed in per cent of the enzyme concentration present in original perfusion fluid (initial value) The amount of carboxypeptidase B is expressed by its kinin destroying activity per ml plasma (A) and also according to the method described by Folk *et al* (1960) (B) (see Methods) The gland was perfused with cat plasma containing a carboxypeptidase B preparation (400 μg of added enzyme protein per ml plasma) The bradykinin *splitting activity of this affluent perfusate* was measured as 280 000 units/*ml* (see Methods) The concentration of carboxypeptidase B in the same solution estimated according to the latter method gave an amount of enzyme equal to 44 250 units (for details see Methods)

levelled out When the common carotid artery was reopened, the level of kinin destroying activity in the effluent blood was lower than that of the previous perfusate, but it remained much higher than the normal value for blood The late recurrence of the vasodilator effect on chorda stimulation could be explained when the carboxypeptidase B activity in venous outflow from the gland and in a gland homogenate was examined at the end of an experiment Even then the venous effluent blood contained considerable amounts of carboxypeptidase B although the gland had been pump perfused with normal plasma for several 10 min periods with intervals of restored normal circulation in between The bradykinin splitting activity in gland homogenates was also found to be increased 3—[illegible] times as compared to the non perfused gland on the other side

In 1 out of 5 cats in this series where the submandibular salivary gland was perfused with normal cat plasma a higher perfusion pressure than usual prevailed Chorda stimulation then resulted in a biphasic vasodilator response In Fig 7 are shown three perfusions with normal cat plasma periods I III and IV and one with carboxypeptidase B containing plasma period II from this experiment The one 10 min perfusion with carboxypeptidase containing plasma abolished the long lasting vasodilator phase which developed after nerve stimulation was finished (period II) The perfusion pressure however had increased By appropriate setting of the pump the perfusion pressure was reduced to a value where the previous chorda induced vasodilatation had levelled out After such adjustment no dilatation was caused by a subsequent chorda stimulation (period III) At a higher (readjusted) inflow pressure one hour later, the biphasic vasodilator response to chorda stimulation had re appeared (period IV) The vasodilator effect of 5 μg isoprenaline injected arterially

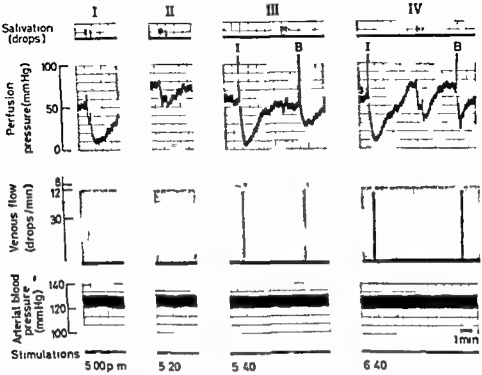

Fig 7 The effect of adding carboxypeptidase B to the perfusate on the vasodilator response to chordo-lingual nerve stimulation in the cat submandibular gland during constant volume in [illegible]

outflow was reduced to 8 drops/min by adjusting the flow pump The perfusion pressure was accordingly reduced as shown. The four (10 min) successive perfusion periods shown were interrupted by intervals of normal circulation through the carotid artery Close arterial injections of isoprenaline, 5 μg at (I) and of bradykinin 3 μg at (B) Stimulation of the chordo-lingual nerve for 30 sec at signals

was the same in periods III and IV while that of 3 μg bradykinin increased somewhat in period IV Between each perfusion normal circulation through the gland was restored as described before

Perfusions of isolated preparation of the lateral gastrocnemius muscle

In order to see if unspecific vascular effects were caused by perfusion of an organ with the different types of perfusates experiments were performed in two cats on an isolated gastrocnemius muscle preparation The gastrocnemius preparation was found to be a suitable control organ as this muscle is known to develop a marked functional hyperaemia Furthermore there is no evidence in favour of kinin formation occurring during muscle contractions (Hilton and Lewis 1958 Webster *et al* 1967) Isolation of the motor nerve and of the arterial and venous blood vessels to the lateral head of

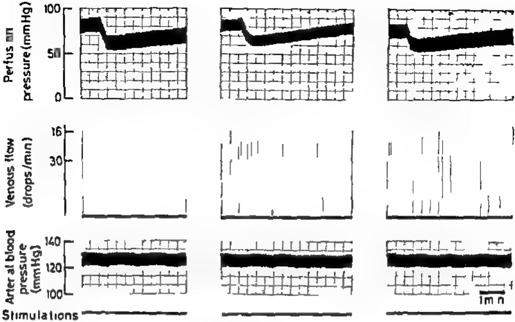

Fig 8 Effects of motor nerve stimulation on circulation in a perfused preparation of the isolated lateral gastrocnemius muscle Constant volume inflow perfusion with red cells suspended in kininogen containing (on left and right) and in kininogen free solutions (middle) (for details see Methods) Stimulation of the motor nerve (5 V 5/sec 1 msec) for 10 sec at signals The twitch contractions elicited were recorded on a kymograph drum and corresponded to the development of 200 g tension Each period of perfusion lasted 10 min normal circulation through the femoral artery being temporarily reestablished for 10 min between them

gastrocnemius was carried out as described in Methods During each test period the femoral artery was clamped and blood pumped into the muscle through a cannulated arterial branch at a constant inflow rate equal to the resting flow in the muscle Vasodilatation was induced by stimulation of the motor nerve causing single twitches or fused tetanic contractions of the muscle preparation as described in Methods When the muscle preparation was perfused with red cells suspended in different types of kininogen containing or kininogen free solutions the vasodilator pattern seen did not change on stimulations of the motor nerve In Fig 8 is shown an experiment of this type where the muscle preparation was perfused for 10 min intervals with red cells suspended in normal cat plasma (to the left and right) and with red cells in a solution containing no kininogen 2 (middle tracing) No change in the vasodilator pattern induced by nerve stimulation was observed on alternation between these perfusates

Motor nerve stimulation at 5/sec was repeated at intervals in muscle preparations perfused with either red cells suspended in kininogen containing or with red cells in kininogen free solutions Still no change in the usual vasodilator pattern could be observed After the first period of nerve stimulation however the vascular resistance often remained at a level somewhat lower than the initial value and a subsequent stimulation would then cause a smaller relative decrease to the same absolute value

When the muscle preparation was perfused with solutions containing carboxypeptidase B in concentrations comparable to those used in the submandibular salivary gland no change in the vasodilator pattern on motor nerve stimulation at 5/sec could be seen When the concentration of carboxypeptidase B in the perfusate was increased to two or three times the amount used in the gland a decrease in the perfusion pressure was observed and a smaller vasodilatation was caused by stimulation of the motor nerve The mediated vasodilator pattern did not change however When the frequency of stimulation was increased from 5/sec to 50/sec an augmented and more long lasting vasodilator response was seen but the general character of the response did not change

Discussion

During perfusion of the submandibular salivary gland with red blood cells suspended in normal cat plasma perfusion pressures of 55—120 mm Hg prevailed The normal arterial pressure in the gland is not known, but must presumably be within this range At such pressures a characteristic type of vasodilator response was maintained on repeated stimulations of the chordo-lingual nerve There occurred an initial rapid decrease in vascular resistance which was followed by a slow recovery of vascular tone to the pre stimulation level The vasodilator response outlasted the nerve stimulation by 5 min or more When perfusates consisting of red cells in solutions containing no kininogen 2 were used two different pictures could be seen depending on the existing inflow pressure

1 When the gland was perfused at a low inflow pressure (below 55 mm Hg) the chorda induced vasodilatation became markedly reduced or was abolished on repetitive nerve stimulation This type of result could be explained if the chorda mediated vasodilatation in a gland perfused at low perfusion pressure was mainly caused by a chemical mechanism which was dependent on the presence in the perfusate of kininogen 2 The reduction in or abolition of the vasodilator response to repeated chorda stimulations could then be the result of depletion of kininogen 2 from the interstitial spaces in the gland tissue

2 When the gland was perfused at a higher inflow pressure (60—120 mm Hg) obtained by increasing the haematocrit value only the initial rapidly occurring vasodilator phase was observed on chorda stimulation Under these conditions the vascular tone returned to its initial level immediately after nerve stimulation was finished Furthermore this type of vasodilator response was not reduced by repeated stimulations

The two different pictures of vasodilator effects on nerve stimulation under these experimental conditions strengthen the concept that two mechanisms are involved in the chorda induced vasodilatation One mechanism would be mediated through the direct action of vasodilator nerve fibres Another mechanism would work through kinin formation occurring secondary to an activation of the gland cells and requiring the presence of kininogen 2 in the perfusate for its maintenance (Gautvik 1970 a)

The biphasic long lasting chorda induced vasodilatation seen when a perfusate of

red cells suspended in normal cat plasma was used changed into the monophasic short lasting type when a kininogen free solution was used The usual biphasic and long lasting vasodilator response to chorda stimulation could only be restored however, when normal circulation through the carotid artery had been re established for about 1 hr Further, the addition of partly purified kininogen 2 to the kininogen free solution perfusing the gland failed to normalize the vasodilator response These findings cannot be fully explained A possible explanation would be that a decreased capillary permeability developed in the vascular bed when the gland was perfused with red cells in kininogen free solution so that a reloading of the interstitial spaces with kininogen 2 would be a slow process

The experiments with horse serum were complicated by the presence of naturally occurring agglutinins against cat red cells These agglutinins had to be removed before the serum could be used in perfusion studies When glands were perfused with cat red cells in such adsorbed horse serum the vasodilatation provoked on chorda stimulation was of the short lasting type Bhoola *et al* (1965) perfused the submandibular salivary gland in cats with horse serum to which had been added eserine They explained the vasodilatation caused by chorda stimulation as due to the effect of dilator nerve fibres Their results do not exclude the possibility, however, that other vasodilator mechanisms may participate in the chorda mediated vasodilatation under more normal conditions The present experiments show that the long lasting phase in the complete vasodilator response is lacking during horse serum perfusions This lacking phase is the one which is connected to kinin formation

Skinner and Webster (1968) injected carboxypeptidase B intravenously into cats in amounts which abolished the effect of close arterial infusions of bradykinin in doses of 1 5—4 μg/min but they did not observe any effect of the increased kinin destroying activity in plasma on the chorda mediated vasodilatation The average blood levels of carboxypeptidase B in their experiments equalled 80—170 μg/ml and may not have been sufficiently high The concentrations of carboxypeptidase B in affluent fluid to the gland in the present experiments was probably 3—8 times higher than those used by Skinner and Webster (1968) During their experiments the gland had its natural blood circulation If high pressures of perfusion existed a moderate effect of carboxypeptidase B might have been difficult to detect

The results presented demonstrate that carboxypeptidase B in some way or other became firmly attached to glandular elements since a high kinin destroying activity persisted in the gland for a long time and thus delayed the reappearance of chorda induced vasodilatation during the perfusion periods with normal plasma The low recovery of carboxypeptidase B in the venous effluent could be explained partly by an uptake into the gland and partly as a result of some instability of this enzyme

When carboxypeptidase B was infused to the gland a reduction in the resting vascular tone was observed The lowering of perfusion pressure which thus developed might again explain the temporary lack of a nervous dilator component in the stimulation response (Gautvik 1970 a) This dilator effect of the enzyme preparation on the vascular bed was apparently abolished when normal circulation had been restored

for some time The temporary reduction of vascular tone seen during infusions of the carboxypeptidase B preparation could be an effect caused by impurities of other enzymes (*i e* trypsin and chymotrypsin) However the carboxypeptidase B preparations purified according to the method of Folk *et al* (1960) have been reported to contain no trace of trypsin chymotrypsin or of carboxypeptidase A

The alternation between red cells in kininogen free and red cells in kininogen containing solutions as perfusates did not change the vasodilator responses of motor nerve stimulations in an isolated gastrocnemius preparation Also carboxypeptidase B present in the perfusate in concentrations comparable to those given to the gland did not change that vascular response This last finding confirms those of Webster *et al* (1967) who found that carboxypeptidase B had no effect on the functional vasodilatation in the perfused gracilis muscle of the dog

The present experiments strongly indicated that although a direct dilator nerve effect may cause a powerful vasodilatation in the submandibular salivary gland, an additional kinin dependent mechanism is also part of the complete vascular response to chordo lingual nerve stimulation There is no contradiction in the functional hyperaemia of glands involving two vasodilator mechanisms A nervous mechanism would ensure an immediate increase in flow Release of kinin forming enzymes (kallikrein(s)) during activation of glandular elements would make possible a vasodilator mechanism linked to gland activity and suitable for adjustment of local flow according to metabolic needs as first suggested by Hilton and Lewis (1955)

The serological work was most kindly carried out by Dr Else Vogt at National Institute of Public Health Oslo The author wants to thank Mojmir Kriz for skilful technical assistance and extensive cooperation throughout the work This project has benefited from financial support to the Institute of Physiology from the Norwegian Research Council for Science and Humanities from the Nansen Foundation and from the Norwegian Council on Cardiovascular Diseases This support is gratefully acknowledged

References

BHOOLA K D J MORLEY M SCHACHTER and L H SMAJE Vasodilatation in the submaxillary gland of the cat *J Physiol* (Lond) 1965 *179* 172—184

FOLK J E K A PIEZ W R CARROLL and J A GLADNER Carboxypeptidase B IV Purification and characterization of the porcine enzyme *J biol Chem* 1960 *235* 2272—2277

GAUTVIK K Kininogen in plasma from cat hamster and guinea pig *Acta physiol scand* 1969 *76* 191—200

GAUTVIK K Studies on kinin formation in functional vasodilatation of the submandibular

C tation in the

C in the submandibular salivary gland when activated by chorda stimulation *J Physiol* (Lond) 1970 (To be published)

HILTON S M and G P LEWIS The cause of the vasodilatation accompanying activity in the submandibular salivary gland *J Physiol* (Lond) 1955 *128* 235—248

HILTON S M and G P LEWIS Vasodilatation in the tongue and its relationship to plasma kinin formation *J Physiol* (Lond) 1958 *114* 532—540

JACOBSEN S Separation of two different substrates for plasma kinin forming enzymes *Nature* (Lond) 1966 *210* 98—99

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WEBSTER M E N S SKINNER and W J POWELL Role of the kinins in vasodilatation of skeletal muscle of the dog *Amer J Physiol* 1967 *212* 553—558

Acta physiol scand 1970 79. 204—215

From the Institute of Physiology, University of Oslo, Norway

Parasympathetic Neuro-Effector Transmission and Functional Vasodilatation in the Submandibular Salivary Gland of Cats

By

KAARE GAUTVIK

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Abstract

GAUTVIK K. *Parasympathetic neuro effector transmission and functional vasodilatation in the submandibular salivary gland of cats* Acta physiol scand 1970 79 204—215

The present study deals with the effect of different pharmacological agents on the secretory and vasodilator responses caused by stimulation of the chordo-lingual nerve in cat submandibular salivary glands perfused under conditions of constant volume inflow

When the gland was perfused with red cells suspended in normal cat plasma or in kininogen-free solutions the vasodilator and secretory responses caused by stimulation of the chordo-lingual nerve were mimicked by close arterial injections of acetylcholine Atropine injected intravenously in doses of 0 5—1 mg/kg did not affect the chorda induced vasodilatation while salivary secretion was blocked *Increasing doses of atropine caused a successive reduction in the* chorda mediated vasodilatation

Close arterial injections of isoprenaline caused vasodilatation *but no secretion of saliva* Propranolol in doses of 10—200 μg infused into the gland abolished the effect of injected isoprenaline but caused a small reduction of the chorda mediated vasodilatation The combined action of propranolol and atropine depressed the long lasting vasodilator phase of chorda stimulation while the initial decrease in perfusion pressure was not affected

The findings suggest that the chordo-lingual fibres to this gland all are cholinergic and that the nerve-effector transmissions vary in their atropine sensitivity The neuroglandular synapse responsible for secretion of watery saliva is easily blocked by atropine The neuro-vascular transmission and the neuro glandular connection responsible for release of kallikrein are both relatively atropine resistant

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In 1872 Heidenhein reported that atropine abolished the secretion of saliva whereas it had hardly any effect on the vasodilatation caused by stimulation of the *chordo-lingual nerve There is no conclusive evidence for the presence* of non cholinergic fibres in that nerve however and later workers found that large doses of atropine were able to abolish also the chorda mediated functional vasodilatation (Barcroft 1914, Dale and Gaddum 1930) Although it is generally accepted that chordo-lingual nerve fibres to the salivary gland are cholinergic (for review see *Emmelin* 1967), the existence of *adrenergic β receptors involved in the chorda induced vaso-*dilatation has been suggested (Skinner and Webster 1968)

The chorda-mediated vasodilatation in the submandibular salivary gland of cat has been studied in two previous publications (Gautvik 1970 a, b). Support was found for this functional vasodilatation being mediated via two different mechanisms, one being a direct action of vasodilator nerve fibres and the other involving kinin formation According to this view, the chorda-evoked vasodilatation in the submandibular gland is brought about through the activation of two different neuro-effector mechanisms namely neuro-vascular transmission and the neuro-glandular connection responsible for release of kallikrein A more detailed study of certain pharmacological aspects of these two neuro-effector mechanisms is the subject of the present paper

Methods and materials

(1970 a)

The haematocrit of the final perfusate entering the gland varied from 25 to 35 per cent

Arterial injections or injections into the perfusion fluid were made through a T-piece inserted in polyethylene tubing cannulating the lingual artery close to the gland

Intravenous injections were made through a cannula in the femoral vein *Femoral arterial blood pressure* was recorded by a Statham transducer (P 23 De) *Heparin* (Novo) 500 I U /kg was injected i v before cannulations were carried out Repeated *injections of heparin* (500 I U /kg) were carried out every second hr

In this investigation the term kininogen 2 is used synonymously with the term substrate II as the substrate for glandular kallikrein

Perfusates used

1 *Heparinized cat plasma* and

2 *kininogen-free perfusates* were prepared as described by Gautvik (1970 a)

3 *Suspension of red blood cells* A nearly pure stock suspension of red cells was obtained from heparinized cat blood as described earlier (Gautvik 1970 b) Perfusates containing red cells were prepared from the red cell stock suspension by addition of perfusates 1, 2, 3 or mixtures of these to the desired haematocrit

Before passing the pump the perfusates were heated to 37° C Before being used all non-corpuscular perfusates were filtered through a millipore filter (Millipore Filter Corporation, Bedford, Massachusetts USA) with pore size of 0 22 μ

Salivary secretion The submandibular ducts were exposed in the floor of the mouth and

stimulated supramaximally (9—14 V) with square wave pulses of 1 msec duration and at a frequency of 20 per sec The duration of one period of stimulation was 30 sec unless otherwise stated

Control procedures for evaluation of the functional state of the gland and of its vascular anatomy was performed as described by Gautvik (1970 a)

Determination of kininogen 1 and 2 The amounts of kininogen 1 and 2 in cat plasma and in other kininogen-containing solutions were estimated in terms of bradykinin equivalents that were formed per ml (Gautvik 1969) using the isolated rat uterus preparation for evaluation of kinin activity

Drugs Acetylcholine was used as chloride (S A F Hoffman—La Roche & Co A G) and isoprenaline as sulphate puriss (Siegfried Somgen Switzerland) Synthetic bradykinin (BRS 640 Sandoz, Basel Switzerland) was used Atropine was used as sulphate and propranolol as hydrochloride (Imperial Chemical Industries Ltd)

Results

The haemodynamic and secretory effects of acetylcholine in a gland perfused with red cells in kininogen containing and kininogen free solutions

The complete chorda induced vasodilator response in a gland perfused with red cells suspended in normal plasma has earlier been shown to consist of two phases. The first phase is rapidly initiated and short lasting the other is more slowly developing but lasts for a longer period of time This latter, slowly developing vasodilator effect of chorda stimulation seemed to be caused by kinins formed in the gland and it lasted for 6 min or more (Gautvik 1970 a) If only cholinergic transmissions are involved in the chorda induced vasodilatation then it should be possible to mimic the nervous vasodilator pattern with close arterial injections of acetylcholine In experiments on 9 cats the submandibular glands were perfused at constant inflow rate with red cells resuspended in normal cat plasma and the vasodilator responses to 30 sec supramaximal chorda stimulation were compared to that caused by close arterial injections of acetylcholine In these experiments doses of acetylcholine were used which provoked secretion of saliva

The vasodilator pattern seen on such acetylcholine injections did actually mimic that caused by chordo-lingual nerve stimulation There was a rapid fall in the perfusion pressure which then returned slowly to prestimulation level or stabilized on a lower value The prolonged vasodilator effect of acetylcholine was comparable in time to that seen after chorda stimulation.

The vasodilator responses to arterial acetylcholine injections were compared to those caused by stimulation of the chordo-lingual nerve also in 3 glands which were alternately perfused with red cells suspended in normal plasma and red cells in a kininogen free solution The results were uniform and one typical experiment from this series is shown in Fig 1 In perfusion period I left column the vasodilator and secretory responses to a 30 sec chorda stimulation are shown during perfusion with red cells suspended in normal plasma. In period I right column 0 1 μg of acetylcholine is injected close arterially to the gland during the same perfusion period The chorda stimulation caused here as is often seen a two-phasic vasodilator response with an initial rapid drop in the perfusion pressure After the nerve stimulation had finished a further fall in perfusion pressure was seen and then the vascular resistance returned to the initial value in approximately 7 min Acetylcholine injection (period I right column caused a rapidly occurring vasodilatation compa

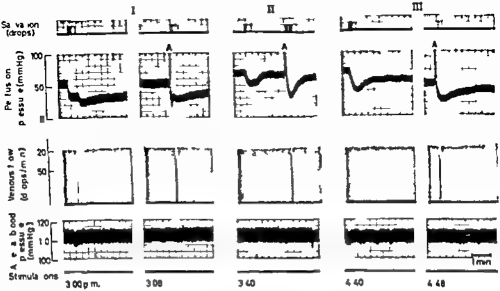

Fig. 1. Vasodilator and secretory responses in perfused cat submandibular salivary gland to stimulation of the chordo-lingual nerve and to acetylcholine injections. Three (15 min) successive perfusion periods are shown interrupted by intervals of normal circulation through the common carotid artery. The gland was perfused at constant volume inflow with red cells suspended in normal cat plasma (periods I and III) and in kininogen free solution (period II) (haematocrit 28 per cent). Stimulations of the chordo-lingual nerve for 30 sec periods at signals. Close arterial injections of acetylcholine 0.1 µg at (A). (For details on the perfusion procedure see Methods.)

rable to that of a 30 sec chorda stimulation. The two responses were also similar in their slow return of perfusion pressure towards the initial value. In period II the vasodilator and secretory effects of chorda stimulation and of acetylcholine injection (0.1 µg) were compared in the same gland, this time perfused with red cells resuspended in a kininogen free perfusate. The vasodilatation caused by chorda stimulation was now relatively short lasting, as was also the vasodilator effect of injected acetylcholine. When the gland was again perfused with red cells in normal plasma, the vasodilator response both to chorda stimulation and to acetylcholine injection were long lasting once more (perfusion period III).

The effect of atropine on the chordo-lingual mediated vasodilatation in the submandibular salivary gland

The submandibular salivary glands of 8 cats were perfused at constant flow rate with red cells resuspended in normal plasma, and the effect of atropine on the vasodilator response to a 30 sec supramaximal chorda stimulation observed. Fig. 2 demonstrates results from 5 successive perfusion periods in one typical experiment where atropine was given in successive increasing doses. The venous outflow from the gland was constant and equal to 0.3 ml/min during the perfusion periods. In

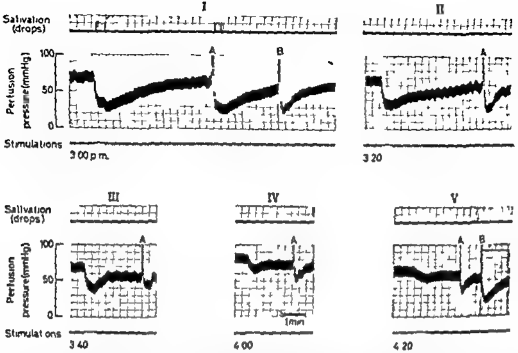

Fig 2 Vasodilator and secretory responses in perfused submandibular gland to stimulation of the chordo-lingual nerve and to injections of acetylcholine and bradykinin The gland was perfused at constant volume inflow with red cells suspended in normal cat plasma at a haematocrit of 33 per cent Five successive perfusion periods were presented interrupted by intervals where normal circulation through the carotid artery was re established Before period II, 1 mg/kg of atropine sulphate had been injected intravenously In perfusion periods III IV and V, 0 15 mg 0 85 mg and 1 6 mg of atropine sulphate had been infused into the gland respectively Stimulations of the chordo-lingual nerve for 30 sec periods at signals Close arterial injections of acetylcholine, 0 15 μg at (A) and of bradykinin, 0 1 μg at (B)

period I is seen the usually long-lasting vasodilator response caused by a chorda stimulation and the similar vasodilator effect of an acetylcholine injection (0 15 μg). Also the effect of bradykinin (0 1 μg) injected close arterially to the gland is shown Atropine (1 mg/kg) was then given intravenously to the animal during a 10 min period where normal circulation was restored After this dose of atropine, the vasodilator pattern caused by chorda stimulation could not be seen to have changed (period II) The vasodilator effect of acetylcholine injection (0 15 μg) was however, reduced and the production of saliva to both stimuli completely abolished Periods III, IV and V demonstrate the effects of 3 successive doses of atropine (0 1 mg/ml perfusate) infused directly into the gland The accumulated doses of atropine given to the gland before the three chorda stimulations of periods III, IV and V were 0 15 mg, 0 85 mg and 1 6 mg, respectively Such additions reduced progressively the vasodilator response to chorda stimulation Also a gradual change in the vasodilator pattern seen on nerve stimulation could be observed The slope and total decrease in the perfusion pressure were more affected than the duration of the

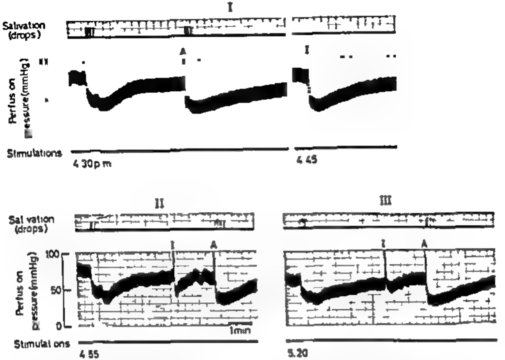

Fig 3 Vasodilator and secretory responses in perfused cat submandibular gland to chorda stimulation and to injections of acetylcholine and isoprenaline The gland was perfused at constant volume inflow with red cells suspended in normal cat plasma at a haematocrit of 33 per cent The three successive perfusion periods shown were interrupted by intervals of normal

response The vasodilator effect of injected bradykinin was not significantly changed while that caused by acetylcholine was markedly reduced in the last three perfusion periods An amount of atropine of 2 mg or more infused into the gland abolished all vasodilator responses to chorda stimulation

The effect of propranolol on the chorda mediated vasodilatation in the submandibular salivary gland

The submandibular salivary gland in 6 cats was perfused with red cells suspended in normal cat plasma to which was added propranolol in different concentrations and the vasodilator response to a 30 sec supramaximal chorda stimulation observed When from 10 to 200 μg of propranolol were infused into the gland in the course of 2 hrs, the chorda induced vasodilatation was depressed to a small extent only In Fig 3 is presented results from such an experiment where the effect of propranolol infused into the gland in increasing doses is observed during stimulation of the

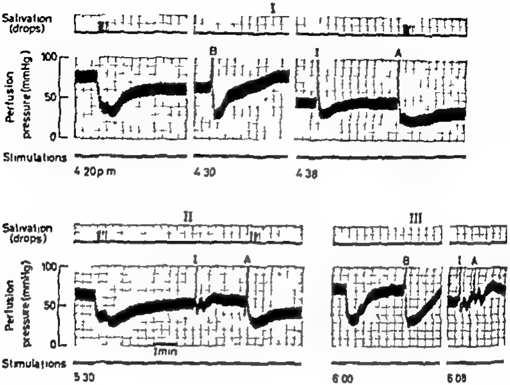

Fig 4 Vasodilator and secretory responses in perfused cat submandibular gland to chorda stimulation and to injections of bradykinin of isoprenaline and of acetylcholine The gland was perfused at constant volume inflow with red cells suspended in normal cat plasma at a haematocrit of 30 per cent The three successive perfusion periods shown were interrupted by intervals of normal circulation through the carotid artery Before period II 40 μg of propranolol was infused to the gland and before period III 1 mg/kg of atropine sulphate was injected IV Stimulation of the chordo-lingual nerve for 30 sec periods at signals Close arterial injections of bradykinin 0 1 μg at (B), of isoprenaline 1 μg at (I) and of acetylcholine 0 1 μg at (A)

chordo-lingual nerve Records of salivary secretion and perfusion pressure are shown during 3 successive perfusion periods using red cells in normal cat plasma as perfusate Period I shows the usual vasodilator response to chorda stimulation as well as the effects of isoprenaline (4 μg) and acetylcholine (0 1 μg) injected close arterially to the gland In periods II and III the effects of the same vasodilator stimuli are shown after infusion of 40 μg and 150 μg of propranolol respectively The initial level of perfusion pressure was somewhat lowered during the infusions of propranolol so that the relative decrease in perfusion pressure caused by chorda stimulation in the last period was reduced The nerve induced vasodilator pattern was however not changed whereas the effect of injected isoprenaline (4 μg) was gradually depressed These doses of propranolol had also a moderate anti cholinergic effect as the duration of the vasodilator response to acetylcholine injections was somewhat reduced

The combined effect of propranolol and atropine on the chorda induced vasodilatation in the submandibular gland

Skinner and Webster (1968) reported that the two drugs given in combination were more effective in reducing the chorda induced vasodilatation than either of them alone In 4 cats the combined action of propranolol and atropine on the chorda provoked vasodilatation was therefore examined and the results were all uniform The glands were again perfused with red cells suspended in normal cat plasma to which propranolol had been added, and arterial circulation was restored between the perfusion periods During these experiments the gland received doses of propranolol (40—100 μg) which had previously been shown to cause no or a small reduction of the chorda mediated vasodilatation only without changing the vasodilator pattern A dose of atropine (1 mg/kg) which alone had no effect on the chorda induced vasodilatation was then given i v The following chorda stimulations resulted in a vasodilatation of a much shorter duration Results from one of these experiments were presented in Fig 4 In perfusion period I are seen the normal vasodilator responses to a 30 sec supramaximal chorda stimulation and to close arterial injections of bradykinin (0 1 μg), of isoprenaline (1 μg) and of acetylcholine (0 1 μg) The chorda induced vasodilator pattern was not changed in period II prior to which 40 μg of propranolol had been infused into the gland The vasodilator effect of isoprenaline was however hardly detectable while that of acetylcholine was not significantly reduced In period III where in addition i v injected atropine (1 mg/kg) had been given chorda stimulation caused a much more short lasting vasodilatation The vasodilator responses to close arterial injections of isoprenaline (1 μg) and to acetylcholine (0 1 μg) were very much reduced Control injection of bradykinin (0 1 μg) showed however an unchanged vasodilator effect

Discussion

The vasodilator effect of chorda stimulation in the gland perfused with red cells resuspended in normal cat plasma has earlier been explained as a result of a direct action of vasodilator nerve fibres assisted by the formation of kinins Kinin formation has previously been shown to be the cause of the long lasting phase in the chorda mediated vasodilatation (Gautvik 1970 a b) In glands perfused alternatively with red cells in kininogen containing and with red cells in kininogen free solutions the effect of acetylcholine injection resembled the vasodilator effect of chorda tympani stimulation. The duration of the vasodilatation caused by chorda stimulation as well as of that caused by acetylcholine injection was reduced when the gland was perfused with red cells in kininogen free solutions where intra glandular formation of kinins must have been very much reduced or abolished As has been described earlier (Gautvik 1970 b) the duration of the vasodilator responses to chorda stimulation and to acetylcholine injection were also reduced in glands perfused with red cells in normal cat plasma containing carboxypeptidase B a kinin inactivating enzyme In this situation where the vasodilatation was short lasting the vasodilator

effect of bradykinin injected close arterially was greatly depressed The present results strongly suggest that the vasodilator effect of injected acetylcholine is mediated partly through its direct action on the vessels and partly through the release of kallikrein from the gland cells leading to kinin formation, as also suggested by Hilton and Lewis (1956) Atropine given i v in doses of 0 5—1 mg/kg had no demonstrable effect on the two vasodilator mechanisms while the secretion of saliva was extinguished The effects of acetylcholine injections, however, were greatly reduced though some vasodilator effect of injected acetylcholine (0 15 μg in 0 15 ml of modified Krebs-Ringer solution) could be demonstrated even in atropinized animals Under the conditions of the present experiments the drug was injected into the tubing close to the gland from where it would reach the glandular vessels as a concentrated bolus Therefore, the vascular bed of the gland was exposed to a high concentration of acetylcholine Furthermore, with the low rate of flow (0 2—0 4 ml/min) the injected drug would pass through the gland in the course of about 30 sec This special experimental situation may well account for the remaining effect of acetylcholine

Moderate doses of atropine were unable to block the vasodilator effect of chorda tympani stimulation This may be due to the release of acetylcholine at the nerve terminals taking place in such a close aproximation to the receptor sites that availability of atropine at the receptor sites is limited, as suggested by Dale and Gaddum (1930) When atropine was infused into the gland in doses of 2 mg or more, however, both neuro vascular and neuro glandular transmission were blocked so that chorda stimulation was not followed by a decrease in vascular resistance ' ' and Beilenson (1968) held the view that the atropine-resistant vasodilatation represents an extreme case of the variation in the sensitivity of cholinergic receptors to this drug (Ambache 1955)

Species differences are apparently involved regarding the sensitivity of the chorda induced vasodilatation towards atropine Morley *et al* (1966) found thus that in the rabbit submandibular gland both secretion and vasodilatation are sensitive to atropine block They concluded therefore that true vasodilator fibres exist in the chorda tympani nerve of this animal

The vasodilator pattern caused by chorda stimulation in the present experiments changed in a typical way when increasing doses of atropine were administered to the gland The slope and maximal decrease in perfusion pressure were more affected than the duration of the vasodilatation The chorda induced vascular response changed towards the one which prevailed during low pressure perfusions and which was thought to be mediated essentially through the chemical vasodilator component (Gautvik 1970 a b) If the action of vasodilator nerve fibres was more affected by atropine than was the neuro glandular transmission responsible for the release of kallikrein, then the duration of the vasodilator response to acetylcholine injections should be less affected than the initial fall in vascular resistance

The present results showed however that the duration of the acetylcholine provoked vasodilatation was more reduced than its initial vasodilator effect This can

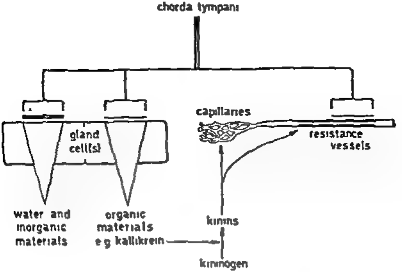

Fig 5 A schematic model for regulation of the vasodilatation caused by stimulation of the chordo-lingual nerve

Vasodilator nerve fibres supplying glandular vessels and secretory fibres innervating glandular elements are suggested The parasympathetic innervation is believed to be cholinergic but the neuro-effector transmissions involved seem to have different sensitivities towards atropine

Symbols

Release of acetylcholine ////////

Blocking effect of atropine very good ▬

poor ═

possibly be explained when considering the method of administering acetylcholine under the present experimental conditions During the perfusion experiments the effective intravascular concentration of the injected acetylcholine was probably much higher than that at the neuro-glandular receptor sites which acetylcholine reached by diffusion The immediate vasodilator effect of acetylcholine exerted directly on the intra glandular vessels could accordingly dominate and the possible difference in atropine resistance between the neuro-vascular and the neuro-glandular connection would thus not be demonstrated

Propranolol (10—200 μg) infused into the gland in the course of 2 hrs could not depress the vasodilatation evoked by chorda stimulation more than could be explained by the anti-cholinergic effect exerted by high doses of this substance The marked vasodilatation caused by injections of isoprenaline close arterially was gradually eliminated when propranolol was administered to the gland

The findings of Davey *et al* (1965) which were later confirmed by Schachter and Beilensen (1968), did not suggest that adrenergic β receptors play a role in the chorda mediated vasodilatation Neither group found a significant change in the vasodilator response evoked by chorda stimulation in cats that had been pretreated with reserpine The present findings support the notion that adrenergic β receptors exist in the vasculature of the gland but they do not support the view of β-receptors being important for the vasodilatation caused by stimulation of chorda tympani

Skinner and Webster (1968) also reported that the combination of propranolol and atropine reduced the chorda-evoked vasodilatation more than each drug alone. Their findings could be confirmed, but in the present experiments the kinin dependent vasodilator mechanism was apparently more affected by the combined action of these drugs than was vasodilatation due to the direct action of nerve fibres. The chemical vasodilator mechanism is presumably dependent upon the release of active kallikrein from the gland cells, the formation of kinins in the interstitial fluid and of the actions of the kinins so formed on a sensitive vasculature. As the vasodilator responses caused by bradykinin injections were unaltered during administration of propranolol and atropine, one explanation for the combined action of these drugs could be that they inhibited release of kallikrein from the gland cells.

In Fig. 5 is suggested a model for regulation of the chorda mediated hyperemia in the submandibular salivary gland. A hypothetical relationship between the two vasodilator mechanisms is shown. The possibility exists that the two vasodilator components act principally at two different sites in the intra-glandular vascular bed. The rapidly initiated and potent vasodilator response following chorda stimulation points to a nervous control of vessels important for the total resistance in the glandular vasculature. The kinins formed in the interstitial fluid may act essentially on other segments of the vascular bed. Besides the vasodilator effect exerted by the kinins released, they will probably assist in increasing capillary permeability, which occurs during chorda stimulation. This increase in permeability will allow an augmented exchange of kininogen and/or kallikrein across the capillary endothelium and the formation of vasoactive peptides is thus further accelerated (Gautvik *et al.* 1970).

Bayliss and Bradford (1885, 1887) suggested from electrophysiological studies that two different cellular elements in the salivary gland were responsible for the secretion of organic material and of watery saliva, respectively. Additional arguments in favour of this concept are summarized by Langenskiold (1941). However, the evidence for the existence of two or more different types of gland cells responsible for the secretion of different components in saliva is not conclusive (Lundberg 1958). The glandular elements in Fig. 5 might therefore represent one gland cell with one or more receptor sites or two cells with receptors that differ in their sensitivity towards atropine. According to the results presented, the parasympathetic transmission to gland cells seems to consist of 2 types of neuro-effector mechanisms which have different atropine-sensitivity as already suggested by Hilton and Lewis (1956). Consistent with this view are the findings of Gautvik *et al.* (1969) that the kallikrein content was reduced in the submandibular gland of atropinized non salivating cats after prolonged chorda stimulation compared to the nonactivated gland on the other side.

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References

AMBACHE, N, The use and limitations of atropine for pharmacological studies on autonomic effectors *Pharmacol Rev* 1955 *7* 467—494

BARCROFT, J, In *The respiratory function of the blood* 1914 1 st ed pp 140—148 Cambridge University Press

BAYLISS, W M and J R. BRADFORD, On the electrical changes accompanying secretion *J Physiol* (Lond) 1885 [redacted] 13P—16P

BAYLISS W M and J F BRADFORD The electrical phenomena accompanying the process of secretion in the salivary glands of the dog and cat *Int Mschr Anat Histol* 1887, *4* 109—136

DALE, H H and J H GADDUM, Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves *J Physiol* (Lond) 1930 *70* 109—144

DAVEY, M J, R. F DAVIES H REINERT ... blocking agents on the submaxillary gla

EMMELIN N, Nervous control of salivary .
Canal Volume II pp 595—615 Wave

GAUTVIK K, Kininogen in plasma from cat, hamster and guinea pig *Acta physiol scand* 1969 *76* 191—200

GAUTVIK, K, Studies on kinin formation in functional vasodilatation of the submandibular salivary gland in cats *Acta physiol scand* 1970 a *79* 174—187

GAUTVIK K, The interaction of two different vasodilator mechanisms in the chorda tympani activated submandibular salivary gland *Acta physiol scand* 1970 b *79* 188—203

GAUTVIK K M S M HILTON and S H TORRES, Consumption of kininogen in the submandibular salivary gland when activated by chorda stimulation *J Physiol* (Lond) 1970 (To be published)

GAUTVIK K M, K NUSTAD and J VYSTAD, Kininogenase activity in the stimulated submandib ... *ab Invest* 1969 Suppl (In press)

HEIDENH ... f die Nerven der glandula submaxillaris
Pflug ...

HILTON ... between glandular activity, bradykinin formation and functional vasodilatation in the submandibular salivary gland *J Physiol* (Lond) 1956 *134* 471—483

LANGENSKIOLD A, Component potentials of the submaxillary gland electrogram and their ... *iol scand* 1941 Suppl 6 1—109
... ls *Physiol Rev* 1958 *38* 21—40
... od latation in the submaxillary gland of the

SCHACHTER M and S BEILENSON Mediator of vasodilatation in the submaxillary gland *Fed Proc* 1968 *27* 73—75

SKINNER N S and M E WEBSTER Kinins beta adrenergic receptors and functional vasodilatation in the submaxillary gland of the cat *J Physiol* (Lond) 1968 *195* 505—519

Acta physiol. scand. 1970. 79. 216—225

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Effect of Age on the Collagen Content of the Normal Rat Myocardium

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Abstract

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The development of the collagenous tissue in the myocardium was studied in rats aged one day to 18 months During the first three weeks a rapid exponential increase of both the heart weight and the total amount of myocardial hydroxyproline occurred although the concentration of total and insoluble hydroxyproline as estimated per mg of dry tissue showed no significant change Thereafter the growth rates of the heart weight and total and insoluble hydroxyproline fell with increasing age The growth rate of total hydroxyproline fell more slowly however and a slight increase in the content of total and insoluble hydroxyproline per dry tissue was noted with increasing age The proportion of neutral salt soluble collagen hydroxyproline in the myocardium dropped from an initial value of 30 per cent of the total hydroxyproline in newborn rats to 10 per cent at the age of three weeks After this the proportion of

functional demand on the growing heart for increased tensile strength

Collagen is the only protein in the organism showing definite age changes A relationship with the general process of ageing has therefore been assumed Physico chemical changes and changes in the chemical and thermic contraction have been demonstrated in collagen fibres of different ages (Chvapil and Hruza 1959 Verzar 1964) Moreover biochemical changes in the tissues such as a decrease in the content of extractable collagen (Buocek *et al* 1958 Kao and McGavack 1959 Kobrle and Chvapil 1962 Wirtschaftler and Bentley 1962) show a relationship with increasing age and the total collagen content in certain tissues has been found to increase with age (Sobel and Marmorston 1956 Clausen 1963)

Very little research has been dedicated to the connective tissue of the heart and its age changes Most of the relevant studies deal with the content of collagen and mucopolysaccharides in the cardiac valves while very few publications deal with the myocardial connective tissue

Evidently, the connective tissue framework of the heart develops at an early foetal stage. Collagen has been detected chemically already on the 3rd day of development in the chick embryo heart by Woessner *et al.* (1967) and acid mucopolysaccharides can be detected at the same stages of development (Gessner *et al.* 1965).

Contradictory results have been reported in studies on the myocardial collagen during ageing. A significant increase of the total collagen content has been demonstrated by Clausen (1963) in the human myocardium and aorta during the foetal period, postnatally a steady increase was found to occur up to the age of 60. However, other investigators (Oken and Boncek 1957, Laves and Correll 1960, Montfort and Perez-Tamayo 1962, Wegelius and v. Knorring 1964) have failed to observe definite postnatal changes in the total collagen content of the human myocardium. Contradictory results have also been reported in studies on laboratory animals. Thus Schaub (1964) observed an increase due to age in the collagen content of the normal rat myocardium, but Kao and McGavack (1959) were not able to demonstrate definite age changes in the total collagen content, nor in the content of alkali soluble collagen in the rat myocardium. Similarly Sobel and Hewlett (1967) noticed no age increase in the collagen content of the dog heart.

The only available investigations on the content of total and soluble myocardial collagen during early development have been performed on chick embryos. Woessner *et al.* (1967) observed an exponential increase in total collagen in the whole chick heart from the 5th day up to the day of hatching, whereas the content of soluble collagen remained more or less constant.

In the present study the content of soluble and total collagen of the myocardium were studied in normal rats from the age of 1 day up to 18 months.

Material and methods

Experimental animals

Albino Wistar male rats were used except in the youngest age groups (1—21 days), where pooled litters were used without separating the sexes. Within each age group one or two series of rats were used, consisting of 13—42 rats in the youngest age groups (1—21 days) and 3—7 rats in the age groups 2—18 months. The animals were reared on a commercial pelleted diet (Hankkija [illegible] experiments on the effect of hyper [illegible] ere used (v. Knorring 1970 b) [illegible] in this age group but they had [illegible] All animals were weighed immediately before sacrifice.

Preparation of the myocardial tissue

The animals were killed by thoracotomy under ether anesthesia. The pulsating heart was dissected out and immediately placed in 37° 0.9 % NaCl solution. The heart was rinsed in clear solution until the fluid was free from blood. Then it was lightly wiped with filter paper free from superfluous saline and the aorta and valves were removed. The heart was then weighed and a sample was taken for the determination of wet weight. This sample was dried in an oven at 60° C for 24 hrs and then placed in an exsiccator and weighed until a constant dry weight was obtained. In the youngest age groups and in certain series the water content was determined from pooled samples owing to scantiness of material.

The remainder of the myocardium from the hearts in the different groups were pooled and the wet weight was determined. In each age group one or two pooled samples were thus

obtained After gross division by scissors, most of the pooled mass (about two-thirds) was homogenized in ice cold 1 M NaCl solution with an Ultra Turrax homogenizer (Janke—Kunkel) for 5×10 sec at 1 min intervals 5 ml saline per g wet weight of pooled myocardial tissue was used in the homogenization When all air bubbles had disappeared from the sample, 1 ml homogenate was pipetted into small weighed glass tubes for the determination of the dry weight of the homogenate

Preparation of the different hydroxyproline containing fractions from the homogenate

In the present procedure 1 M NaCl solution was used for extraction of the soluble fraction (Levene and Gross 1959) Unbuffered 1 M NaCl solution was used in the same way as Kivirikko (1963) used 1 M and Gross (1958) used 0 45 M NaCl solution The remainder of the homogenate was extracted as described by Kivirikko (1963) at +4° C for 24 hrs under occasional vigorous shaking after which the homogenate was centrifuged at 60 000 g for 30 min

After centrifugation the content of *crude 1 M NaCl soluble hydroxyproline (CSH)* was determined in the unfractionated supernatant after direct hydrolysis of 1 ml supernatant with 1 ml 12 N HCl

Total hydroxyproline (TH) was determined after hydrolysis of 1 ml of the original homogenate performed in the same way as the hydrolysis of the supernatant, or directly from the dry weight sample after addition of 2 ml 6 N HCl and after hydrolysis as described below

Insoluble hydroxyproline (ISH) was not determined from the precipitate, which was discarded ISH was calculated as the difference between TH and CSH

As a rule only one pooled sample was determined in each series owing to the scantiness of material In those series in which double pooled samples were used, the deviation from the mean was relatively slight however

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the ethanol-soluble supernatant was measured and evaporated to dryness on a steam bath The sample was then dissolved in a suitable volume of distilled water and filtered until a clear filtrate was obtained This fraction was used for the determination of free hydroxyproline The *ethanol insoluble precipitate was washed 3 times with 80 % ethanol* recentrifuged and dried at 102° C in a test tube Then the sample was eluated twice with 1 ml 6 N HCl and after hydrolysis used for the determination of NSH The samples were hydrolyzed (3 hrs at ° C) in sealed glass ampoules and neutralized as described by Woessner (1961) After neutralization distilled water was added to a volume suitable for the determination of hydroxyproline

The mean water content in the myocardium in the different groups was used for cal

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NaCl was used for homogenization of 1 g

supernatant represented 1 g of wet myocardial tissue This volume was separately determined in each series

Analytical methods

Determination of hydroxyproline

Hydroxyproline was determined by Woessner's method (1961) Method I was used for the determination of total and crude 1 M NaCl soluble hydroxyproline Method II was used for the determination of 1 M NaCl soluble and free hydroxyproline because of the small amount of hydroxyproline contained in these fractions

Determination of the dry weight of the homogenate

A volume of 0 5 or 1 ml homogenate was pipetted into weighed glass tubes The tubes with their contents were weighed and the samples were evaporated to dryness at 100° C transferred to an exsiccator and weighed until a constant weight was obtained The relative dry weight of myocardial tissue per 1 ml homogenate was calculated as indicated by Kivirikko (1963)

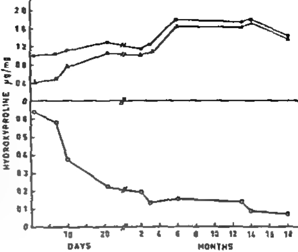

Fig 1 Content of crude 1 M NaCl soluble o, insoluble △ and total ● *hydroxyproline* in the rat myocardium expressed as µg/mg dry weight of the myocardium

The content of insoluble hydroxyproline is calculated as the difference between total and crude 1 M NaCl soluble hydroxyproline

Abscissa age in days or months

Results

Body weight, heart weight and water content of the myocardium

The ratio heart weight/body weight expressed in mg/g decreased with age From the age of one day to 21 days it fell from 5 70 to 4 42 From 2 to 13 months a slow decrease (2 88—2 32) was observed in the heart weight/body weight ratio The deviations within the different age groups from 2 to 18 months were very slight

The water content of the myocardium was highest at the age of one to 21 days (82—80 7 per cent), after which a concentration of 75—78 1 per cent was noted

Content of hydroxyproline in the various fractions of the myocardial homogenate

The concentration of TH, ISH and CSH in the myocardium expressed as µg/mg dry weight of the myocardium are shown in Fig 1

The content of TH showed relatively slight changes from one day up to 3 months of age Somewhat elevated values were observed at 6 to 14 months A slight decrease in the content of TH was noted at 18 months On the other hand, the content of CSH decreased both absolutely as expressed in µg/mg and relatively, as a percentage of TH, from one day to 21 days Subsequently a slow decrease was observed up to the age of 12 months The content of CSH constituted over 60 per cent of TH at the age of one day and only 17 4 per cent at 21 days After this the percentage of TH very slowly dropped with increasing age (Fig 1)

The calculated content of ISH rose up to the age of 3 weeks after which the curve ran parallel with that for TH (Fig 1) The same ISH content was thus noted at 21 days 2 months and 3 months

The amount of hydroxyproline in the myocardium

The age changes in the collagen content can also be expressed as amount of hydroxyproline in the whole myocardium As seen in Fig 2 the increase in wet weight of the

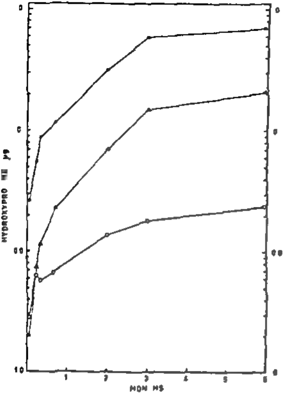

Fig. 2. Heart weights (×) and content of insoluble (△) and crude 1 M NaCl soluble (○) hydroxyproline in the whole myocardium of normal growing rats. Abscissa: age in months.

art and the increase in insoluble hydroxyproline were almost parallel up to nearly 2 months; then the growth rate of both components was reduced. Since the curves have been drawn in semilogarithmic scale, their shape during the first 3 weeks is indicative of an exponential increase in wet weight and insoluble collagen content of the heart.

The slight drop in CSH in the whole myocardium between 7 and 10 days corresponds to the rapid drop in the content per dry weight as shown in Fig. 1.

The amount of free and 1 M NaCl soluble collagen hydroxyproline in the crude 1 M NaCl soluble fraction

A series of rats was investigated in order to determine the proportions of free and 1 M NaCl soluble collagen hydroxyproline in the myocardial CSH at different ages (Fig. 3 A, B).

In the youngest three age groups free hydroxyproline and NSH constituted about half each of CSH (Fig. 3 A). Free hydroxyproline constituted about 25 per cent of CSH at the age of 3 months and only 12 per cent at the age of 6 months and correspondingly NSH constituted about 75 per cent of CSH at the age of 3 months and about [illegible] per cent at the age of 6 months.

When the sum of free hydroxyproline and NSH is compared to the values for

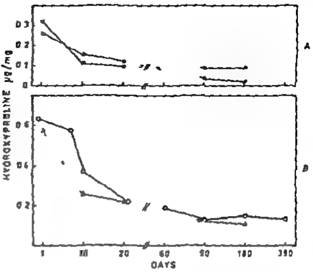

Fig 3 *A* Content of free □ and 1 M NaCl soluble collagen ■ hydroxyproline in the crude 1 M NaCl *soluble fraction of rat myo*cardium
B Content of crude 1 M NaCl soluble ▫ and free + 1 M NaCl soluble ▲ hydroxyproline in the rat myocardium
The values are expressed as μg/mg dry weight of the myocardium Abscissa age in days

CSH obtained in the normal series it appears that this value corresponds fairly well to CSH (Fig 3 B) At one and 10 days this sum was somewhat lower than CSH Free hydroxyproline and NSH were not determined in 7-day-old rats the course of the curve at this level is therefore unknown

Since CSH constituted over half of the total hydroxyproline in the myocardium of 1 day-old rats (Fig 1) NSH and free hydroxyproline constitute about one quarter each of the total hydroxyproline at this age (Fig 3 A) At the age of 6 *months the* content of free hydroxyproline constituted 1 3 per cent and NSH about 5 7 per cent of the mean content of total hydroxyproline as shown in Fig 1

Discussion

The *present results show that in a mechanically functioning* organ such as the heart the amount of collagen increase from birth up to the age of 13—14 months at about the same rate as the total heart weight The most rapid increase in both heart weight and insoluble hydroxyproline in the heart was observed during the period 1—21 days (Fig 2) During this time the growth curves for the wet weight of the *heart and ISH ran parallel courses and the concentration of TH* thus showed little changes Slowly increasing concentrations of ISH were noted up to the age of 6—14 months which indicates that with increasing age ISH constitutes a slowly rising proportion of the myocardial collagenous framework This could be a response to the functional demand on the growing heart for increased tensile strength A slight decrease in *the content of total hydroxyproline was observed in 18 month* old rats A decrease of both the soluble and the insoluble collagen has also been observed in other tissues in old rats *e g* the skin and bone (Laitinen 1967)

During the first week the absolute amount of CSH in the heart increased at the

same rate as the amount of ISH and the heart weight but during the period 7—21 days the amount of CSH remained almost unchanged (Fig 2) The last mentioned observation was obviously due to the marked decrease in the concentration of CSH during this time

These results are in good agreement with those reported in studies on the collagenous framework of the myocardium (Herrmann and Barry 1955) and the whole heart of the chick embryo (Woessner *et al* 1967) The increase in heart weight and total collagen of the whole heart during the period 5—21 days has been described as a continuous exponential function (Woessner *et al* 1967) The growth rate of the heart weight and of total collagen in the heart is much greater in the chick embryo than in the rat during the postnatal period and it is therefore difficult to compare these two animal species

The neutral salt soluble collagen fractions represent the youngest forms of collagen and are thus precursors to insoluble collagen (Gross 1958 Jackson and Bentley 1960) In this study 1 M NaCl was used mainly owing to the scantiness of material The result of determination of 1 M NaCl soluble collagen corresponds fairly well to the amount of the most recently synthetized collagen in a tissue even though slightly older collagen is included (Jackson and Bentley 1960 Kivirikko 1963) NaCl soluble collagen is derived not only from newly formed collagen but also from the catabolism of insoluble collagen (Jackson 1957 Laitinen 1967) Free hydroxyproline is mainly derived from degradation of collagen (Green and Lowther 1959 Prockop *et al* 1962 Kivirikko 1963) and it is assumed mostly to be derived from the ‹br›akdown of soluble collagen in tissues in which a rapid synthesis of new collagen ‹ta›kes place (Kivirikko 1963 Hurych and Chvapil 1965) Up to the age of 3 weeks the crude 1 M NaCl soluble hydroxyproline fraction was found to consist of approximately equal amounts of 1 M NaCl soluble collagen and free hydroxyproline Then the content of free hydroxyproline rapidly decreased Kivirikko (1963) found that half of the total hydroxyproline in 2 day old chick embryos consisted of equal amount of free and 1 M NaCl soluble collagen hydroxyproline Up to the age of 14 days the fractions were equal then the content of free hydroxyproline dropped more rapidly The sum of NSH and free hydroxyproline was found to correspond fairly well to the content of CSH (Fig 3 B) During the first 10 postnatal days this calculated sum was somewhat lower than the content of CSH This may have been due in part to the loss of material during extraction and eluation when these fractions were isolated in part to the presence of ethanol soluble peptide hydroxyproline The content of peptide hydroxyproline was however assumed to be very low (Kivirikko 1963) and was therefore not determined

On the basis of the observations cited above the high content of free and 1 M NaCl soluble collagen hydroxyproline noted in the myocardium during the neonatal period may be interpreted as evidence of a rapid synthesis of soluble collagen during this period During the second to third weeks an increase of the insoluble hydroxyproline content and a simultaneous decrease of the content of 1 M NaCl-soluble collagen and free hydroxyproline were observed It may be assumed that rapid forma

tion of insoluble collagen takes place during this period Recently Heikkinen and Kulonen (1968) and Heikkinen (1968) have shown that the transformation of 0 45 M NaCl soluble collagen into insoluble forms occurs faster in the skin of young rats than in the skin of adult rats However, it has also been suggested by Heikkinen (1968) that a large proportion of the insoluble collagen in the skin of young rats consists of insoluble protein polysaccharide complexes and that only a small proportion consists of intra and intermolecularly linked collagen components, whereas in adult rats the insoluble collagen is mainly characterized by a mutual interaction of peptide chains

Similarly, Jackson and Bentley (1968) have emphasized the possibility that acid mucopolysaccharides and mucoproteins participate in the formation of insoluble collagenous matrices, and Wood (1960) suggested that chondroitin sulphate A/C accelerates the fibrillogenesis of collagen Thus it may be thought that the binding of collagen components to protein polysaccharide complexes which form insoluble matrices contributes to the rapid decrease in the soluble/insoluble hydroxyproline ratio in the rat myocardium during the second to third weeks However, it has been found (v Knorring 1970 a), that the total content of acid mucopolysaccharides and apparently the relative content of the different MPS components in the rat myocardium undergo very slight changes from the age of 7 days to 1 month Therefore, the rapid decrease in the content of soluble hydroxyproline during this period, which was observed in the present study, can hardly depend on a simultaneous rapid increase in the content of chondroitin sulphate

McGavack and Kao (1960) observed no obvious decrease of alkali soluble collagen in the rat myocardium from the age of 3 weeks to 8 months whereas they observed such a decrease in the aorta uterus and cartilage Unfortunately they did not investigate any younger age groups Schaub (1964) who likewise studied the rat myocardium observed an increase of total collagen in ageing animals and a simultaneous decrease of 'labile collagen' which was extracted with Ringer solution at 65° C Schaub (1963) also observed that this decrease in soluble collagen in all rat tissues from birth to adult age was most rapid in parenchymatous organs He assumed that the curve for the decrease was organ specific Gerber and Gerber (1960) also assumed that the process of collagen maturation was organ specific and dependent, for instance, on the function of the collagen in the respective organ It has thus been found that in chick embryos the collagenous framework develops more rapidly in an active organ such as the myocardium than in an inactive organ such as the skin (Woessner *et al* 1967) In the present study the collagenous connective tissue of the rat myocardium was found to grow rapidly during the first three weeks of life Subsequently its composition underwent relatively slight changes These findings are consistent with the results cited above

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References

Bolcek, R. J N L Noble, K-Y T Kao and H R Elden, The effects of age, sex and

cardium and skin *Lab Invest* 1963 *12* 538—542

Gerber, G, G Gerber and K I Altman, Studies on the metabolism of tissue proteins I Turnover of collagen labelled with proline -U- ^{14}C in young rats *J biol Chem* 1960 *235* 2653—2656

Gessner I [redacted] A Lorincz and [redacted] Boström, Acid mucopolysaccharide content of the cardiac jelly of the chick embryo *J exp Zool* 1965 *160* 291—298

Green, N M and D A Lowther, Formation of collagen hydroxyproline *in vitro* *Biochem J* 1959 *71* 55—66

Gross, J Studies on the formation of collagen I Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue *J exp Med* 1958 *107* 247—263

Heikkinen E Transformation of rat skin collagen With special reference to the ageing process *Acta physiol scand* 1968 Suppl 317

Heikkinen, E and E Kulonen, Effect of age on the maturation of rat skin collagen *Biochim biophys Acta* (Amst) 1968 *160* 464—466

Herrmann H and S R Barry, Accumulation of collagen in skeletal muscle, heart and liver of the chick embryo *Arch Biochem* 1955 *54* 526—533

Hurych J and M Chvapil Influence of chelating agents on biosynthesis of collagen. *Biochim biophys Acta* (Amst) 1965 *97* 361—363

Jackson D S Connective tissue growth stimulated by carrageenin I The formation and removal of collagen *Biochem J* 1957 *65* 277—284

Jackson D S and J P Bentley On the significance of the extractable collagen *J biophys biochem Cytol* 1960 *7* 37—42

Jackson D S and J P Bentley, *Collagen glycosaminoglycan interactions* In Gould B S (ed) Treatise on Collagen Biology of collagen London, Academic Press 1968 *2A* 189—214

K Y T and T H McGavack Connective tissue I Age and sex influence on protein omposition of rat tissue *Proc Soc exp Biol* (N Y) 1959 *101* 153—157

o K, Hydroxyproline-containing fractions in normal and cortisone treated chick embryos *Acta physiol scand* 1963 Suppl 219

on Knorring J The mucopolysaccharides in the myocardium of growing rats *Acta physiol scand* 1970 a *79* 226—237

von Knorring J Myocardial collagen in hyper and hypothyroid rats and guinea pigs *Ann Med exp Fenn* 1970 b In press

Kobrle V and M Chvapil The amount of ultrafiltrable and collagenbound hydroxyproline in different organs of the rats during aging *Physiol bohemoslov* 1962 *11* 233—248

Laitinen O The metabolism of collagen and its hormonal control in the rat *Acta endocr* (Kbh.) 1967 Suppl *120*

Laves W and H Correll Alter und Bindegewebehalt des menschlichen Herzmuskels *Dtsch Z ges gerichtl Med* 1960 *50* 643—673

Levene C I and J Gross Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor) *J exp Med* 1959 *110* 771—789

McGavack T H and K Y T Kao The influence of age and sex on soluble collagen in soluble collagen and elastin of rat tissue *Exp Med Surg* 1960 *18* 104—123

Montfort T and R Perez Tamayo The muscle-collagen ratio in normal and hypertrophic human hearts *Lab Invest* 1962 *11* 463—471

Oken, [redacted] E and R J Bolcek Quantitation of collagen in human myocardium *Circulat Res* 1957 *5* 357—361

Prockop, D J B Peterkofsky and S Udenfriend Studies on the intracellular localization of collagen synthesis in the intact chick embryo *J biol Chem* 1962 *237* 1581 1584

Schaub, M C Qualitative and quantitative changes of collagen in parenchymatous organs of the rats during ageing *Gerontologia* (Basel 1963 *8* 114—122

Schaub, M C, The ageing of collagen in the heart muscle *Gerontologia* (Basel) 1964 *10* 38—41

Sobel, H and M J Hewlett Effect of age on hyaluronic acid in hearts of dogs *J Geront* 1967 22 196—198

SOBEL H and J MARMORSTON, The possible role of the gel fibre ratio of connective tissue in the ageing process *J Geront* 1956 *11* 1—7

WEGELIUS O and J VON KNORRING The hydroxyproline and hexosamine content in human myocardium at different ages *Acta med scand* 1964 Suppl *412* 233—237

VERZAR, F, Factors which influence the age reaction of collagen in the skin *Gerontologia* (Basel) 1964 *9* 209—221

WIRTSCHAFTLER, Z T and J P BENTLEY, The influence of age and growth rate on the extractable collagen of skin of normal rats *Lab Invest* 1962 *11* 316—320

W [illegible]

W [illegible]

WOOD G C, The formation of fibrils from collagen solutions 3 Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation *Biochem J* 1960 *75* 605—612

Acta physiol. scand 1970 79. 226—237

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The Mucopolysaccharides in the Myocardium of Growing Rats

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Abstract

VON KNORRING J *The mucopolysaccharides in the myocardium of growing rats* Acta physiol scand 1970 79 226—237

No change in the myocardial mucopolysaccharide (MPS) content, measured as uronic acid of alkali-extracted tissue or hexosamine in papain digests of fresh tissue was observed in rats from the age of 7 days to 18 months When MPS from papain digested myocardial and aortic tissue was separated by electrophoresis on cellulose acetate, three Alcian blue positive fractions were demonstrated Identification of the fractions was attempted on the basis of their electrophoretic mobility their response to testicular hyaluronidase and autoradiography of ^{35}S-containing fractions The slowest fraction (fraction 1) was designated hyaluronic acid (HA), fraction 2 was thought to be identical with heparitin sulphate (HS) and fraction 3 with chondroitin sulphate A/C (CS-A/C) Fractions 1 and 2 in myocardium and aorta had the same electrophoretic properties while fraction 3 from the two tissues differed in this respect As calculated from photoscanned strips HA was predominant in the myocardium and the HS like fraction in the aorta The HA peak showed a slight proportional increase in the myocardium with increasing age corresponding to a decrease in CS A/C The results suggested that the MPS pattern of the myocardium is definitely formed at an early foetal stage and postnatally undergoes only slight age changes

During the last few years both quantitative and qualitative changes associated with ageing have been observed in the connective tissue mucopolysaccharides (MPS) of various organs Particular attention has been paid to age related changes in the MPS pattern in the skin aorta and cartilage A decrease in the amount of MPS, determined as uronic acid or hexosamine has been observed with increasing age in the skin (Loewi and Meyer 1958 Schiller and Dorfman 1960 Clausen 1962)

A decrease with increasing age has also been noticed in the relative amount of uronic acid and hexosamine in dry aortic tissue (Kirk and Dyrbye 1957 Clausen 1962), but the absolute amount of acid mucopolysaccharides (AMPS) appears to remain constant or increase with ageing (Bottcher and Klynstra 1962 Bertelsen 1962) A relative increase in chondroitin sulphate B (CS B) and heparitin sulphate (HS) has been observed in the human aorta while hyaluronic acid (HA) and chondroitin sulphate A/C (CS A/C) decreased (Kaplan and Meyer 1960)

The MPS pattern in the heart valves has been the object of many investigations

(Boström *et al* 1963 Torii *et al* 1965 and Lowther *et al* 1967 As regards the effect of age, the results are in part contradictory and the changes observed have mainly been qualitative

In the myocardium MPS occur not only in the ground substance of the loose interstitial connective tissue, but also in the connective tissue of the vascular walls, the basal membrane of the muscle fibres and round major muscle bundles where the connective tissue forms a coherent framework together with collagen

Very little research has been dedicated to the myocardial MPS composition and its possible age related changes Sulphated MPS are synthetized in the myocardium at an early foetal stage *In vitro*, uptake of ^{35}S has been observed in the myocardium of 3 day-old chick embryos (Gessner and Boström 1965), indicating a synthesis of sulphated MPS already at this stage A decreased ^{35}S incorporation in the human and canine myocardium with increasing age indicative of a reduced synthesis of sulphated MPS, was observed *in vitro* by Hauss and Junge-Hulsing (1961), but to the best of the present writer s knowledge, no corresponding qualitative investigations on age related changes have been reported

Determinations of the amount of MPS in ageing myocardium have given contradictory results Clausen (1962) reported a significant decrease in both uronic acid in the hexosamine in dry myocardial tissue with increasing age, while no age changes in the hexosamine content were observed by Wegelius and v Knorring (1964) in human myocardium

In the present study the uronic acid and hexosamine content in the myocardium and the relative content of the various electrophoretically separated myocardial MPS components was investigated in normal rats of various ages

Material and methods

Animals and preparation of tissue samples

Pooled dry samples of myocardial tissue from normal male albino Wistar rats of various ages were used for extraction of uronic acid. In the youngest age groups (7—21 days) pooled litters were used without separating the sexes The dry myocardial samples had been stored at −22° C until they were analysed for uronic acid In each age group pooled samples from one or two different series were used taken from 13—16 hearts in the age groups 7—21 days and 3—7 hearts in the older groups (3—14 months)

In those series in which the myocardial MPS were electrophoretically separated fresh blood free tissue was used The preparation of these samples has been described in a previous paper (v Knorring 1970 a) The valves based of the aorta and pericardium had been dissected out from all hearts 0 5 g fresh myocardial tissue was taken from each heart Because of the small amount of material available it was necessary to use a pooled sample of 0 5 g in the 7 day group and two pooled samples of 0 5 g each in the 1 month group In addition samples of pooled aorta from 1 and 8 month-old rats were electrophoretically analysed for MPS After removal of the adventitia, 0 2 g pooled aortic tissue was used for this purpose as indicated below

Extraction of mucopolysaccharides (MPS)

1 *Alkaline extraction*

For analys [illegible] 0—80 mg dry myo- [illegible] *et al* 1958 The [illegible] 1 ml MPS extract [illegible]

2. Enzymatic papain digestion

Fresh myocardial and aortic tissue was digested with papain to produce an MPS extract for electrophoretic separation of
ley's method (1965) Papair
of 1 25 mg/0 5 g wet tissue
and dissolved again in 0 4—
same way. The MPS concen

Analysis of hexosamine and uronic acid

Uronic acid was determined by Dische's (1947) carbozole method in MPS concentrates of alkali extracted dry myocardial tissue

Hexosamine was determined by Boas' (1953) modification of Elson and Morgan's (1933) method in dry myocardial tissue and in MPS concentrates of papain-digested myocardium In this analysis, 20—40 mg dry myocardial tissue or 0 1 ml MPS concentrate was used

Electrophoretic separation of MPS

Electro ... il and aortic tissue
was ca ... (1963) The MPS
solution ... ing 20×5 cm Two
sample ... t and a mixture of
known ... d chondroitin sul-
phate ... CS-B (Hoffmann—
La Ro ... l hyaluronic acid=
HA (LEO, Halsingborg, Sweden)

A standard electrophoresis apparatus (LKB) intended for paper and immune electro-
... tion was established
... was carried out at
... ionic strength of
... used The running
... were stained for

... and scanned with
... 50 nm The count
obtained from each peak was expressed as a percentage of the total count

Hyaluronidase digestion

Hyaluronidase solution (0 1 ml) containing 500 TRU testicular hyaluronidase (Hyalas, LEO Halsingborg, Sweden) in 0 9 % NaCl was added to 0 1 ml MPS extract of papain digested myocardium or aorta Digestion was carried out as described by Manley (1965)

Desoxyribonuclease (DNAase) digestion

0 02 mg DNAase (DNAase pancreas, lot 32036, activity 27700 dornase U/mg, Calbiochem,
... ml MPS extract of papain-
... were then precipitated with
... 4° C overnight The samples
... itate was dissolved in 0 1 ml
distilled water for renewed electrophoresis

Autoradiography

Normal rats were sacrified 48 hrs after intraperitoneal injection of 1 5 μC/g b w of ^{35}S sulphate (The Radiochemical Centre Amersham) Isolation of myocardial MPS was carried out as described above and the MPS components were separated by electrophoresis in the
sa
w
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F
35

TABLE I Content of uronic acid and hexosamine in normal rat myocardium

Age	Carbazole uronic acid* (alkaline extraction) µg/mg dry weight	Hexosamine** µg/mg dry weight
7 days	0 38	—
10 »	0 26	—
21 »	0 21	—
3 months	0 28	—
	0 41	—
6 months	0 40	2 52
	—	2 10 ± 0 29
14 months	0 35	—
	0 47	2 68

* Double determinations of a single pooled sample
** Mean ± S D

Electrophoretic separation of 1 M NaCl soluble and 2 N MgCl₂-Soluble AMPS from crude MPS extract

AMPS from MPS extra
precipitated with 0 5 %
tion at 20 000 rpm for 1(
natant with 6 vols ethan
the 2 N $MgCl_2$-soluble
ethanol The two AMPS
separation

Results

The uronic acid and hexosamine content in dry myocardial tissue

Carbazol uronic acid was determined in samples of pooled dry alkali extracted myocardial tissue from rats of various ages (Table I)

A slight decrease in the content of uronic acid was noted from 7 to 21 days of age but thereafter no clear trend was observed The content of hexosamine was only determined in 6- and 14 month old animals No decrease was noted in the older group

Not even in the content of total hexosamine in the crude MPS extracts of papain-digested myocardium were any age variations observed (Table II) The amount of hexosamine in these extracts expressed as µg/mg dry weight of myocardium was approximately half that obtained on direct determination in dry tissue (Table I)

Identification of the electrophoretically separated MPS fractions

1 *Electrophoretic separation of MPS in alkaline buffer solution (pH 9 2)*

Three Alcian blue positive fractions were invariably obtained on separation of MPS extracts from both myocardium and aorta (Fig 1)

TABLE II Hexosamine content in crude MPS extracts of papain digested myocardium of normal rats

Age	Number of hearts	Number of samples	Hexosamine µg/mg dry weight ± SD
7 days	8	1	1.1*
1 month	5	2	0.97 ± 0.06**
8 months	5	5	1.02 ± 0.06
10 months	5	5	1.05 ± 0.17

* Mean value

** Mean deviation

Mean weights of the rats were 7 d 10.5 g 1 month 75 g 8 months 253 g 10 months 312 g

Mean water content of the myocardium were 7 d 81 % 1 month 77.5 % 8 months 76.2 % 10 months 75.1 %

The slowest fraction (fraction 1) migrated with the HA band in the reference sample and was completely digested by testicular hyaluronidase (Fig 2) in all extracts from both myocardium and aorta Fraction 1 was therefore considered as identical with HA

The most rapidly migrating fraction (fraction 3) in both myocardium and aorta had approximately the same mobility as the CS-B reference (Fig 1) However, like the CS-A/C reference fraction 3 from the myocardium was completely, fraction 3 from the aorta almost completely digested by testicular hyaluronidase (Fig 2), whereas the CB S and HS references were completely resistant to this enzyme

Fraction 2 the intermediate band in Fig 1, from both myocardium and aorta

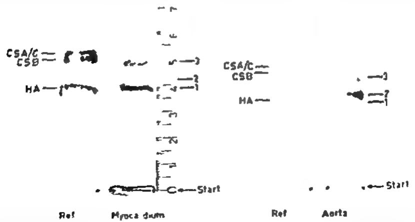

Fig 1 Electrophoretic mucopolysaccharide patterns from the myocardium and from pooled aorta of normal 10 month-old rats *Electrophoresis on cellulose acetate in sodium barbiturate buffer of pH 9.2 at 20 V/cm* running time 80 min 1—3 = different Alcian blue positive fractions Ref = reference substances

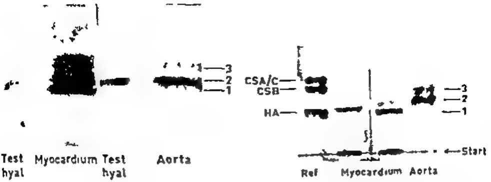

Fig 2

Fig 3

Fig 2 Electrophoretic patterns of myocardial and aortic mucopolysaccharides of normal 10 month-old rats before and after digestion of the samples with testicular hyaluronidase (Test hyal) 1—3 = different electrophoretic fractions Details of electrophoresis as in Fig 1

Fig 3 Electrophoretic patterns of mucopolysaccharides from the myocardium and pooled aorta of normal 8 month old rats Ref = references Pyridine formic acid buffer of pH 2 6, ionic strength 0 05 at 20 V/cm and running time 80 min Fraction 3 from myocardium not visible, probably fused with fraction 2

migrated between the HA and CS B bands in the reference sample and was completely resistant to testicular hyaluronidase (Fig 2) Its mobility was also somewhat slower than that of the HS reference which migrated with the CS B reference

On electrophoretic separation of the 1 M NaCl soluble AMPS obtained after CPC precipitation in 1 M NaCl from the crude MPS extracts all of the 3 previously identified fractions could be demonstrated A weak band with the same mobility as fraction 3 was demonstrated as the only 2 N $MgCl_2$ soluble component

In alkaline buffer solution, contamination with nucleic acids (desoxyribonucleic acid DNA, and ribonucleic acid RNA) may make the identification of CS A/C and CS B more difficult (Franco Bowder *et al* 1963) Photoscanning of electrophoretically separated myocardial MPS extracts before and after DNAase digestion revealed no relative decrease of the three fractions indicative of contamination with DNA

Nucleic acids absorb ultraviolet light to a marked degree However, when samples of MPS solutions from myocardium were applied to unstained cellulose acetate strips and examined in ultraviolet light no absorption was observed The DNA reference on the other hand absorbed ultraviolet light in high degree

When strips were stained with nigrosin protein was observed at the base line and in fraction 1 but not in fractions 2 and 3

2 *Electrophoretic separation in acidic buffer solution (pH 2 6—4 8)*

In acidic buffer (pyridine formic acid pH 2 6—3 6), fraction 1 still migrated with the HA reference and a band with the same mobility as fraction 2 migrated between the CS B and HA references (Fig 3) and somewhat slower than the HS

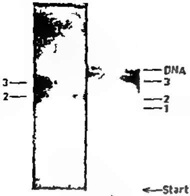

Fig 4

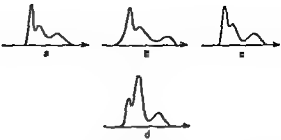

Fig 5

Fig 4 ^{35}S autoradiograph of myocardial mucopolysaccharides of a normal 8 month-old rat a = unstained part of the cellulose acetate strip b = the Alcian blue stained part of the same strip Fractions 1—3 correspond to those in Fig 1, the fourth fraction is probably DNA (see text) Details of electrophoresis as in Fig 1

Fig 5 Chromoscan patterns of electrophoretically separated mucopolysaccharides from myocardium (a—c) and aorta (d) of normal rats of different ages Age of the rats a 7 days b 1 month c and d 10 months Note the difference between patterns of a—c and d Electrophoretic details as in Fig 2

reference However even at pH 2 6—3 6 the HS and CS B references could not be separated from each other At these buffer pH values fraction 3 was not detected the myocardial extracts while in the aortic MPS this fraction could still be demonstrated at the CS B band (Fig 3) At pH 4 8 all 3 fractions could again be demonstrated in all myocardial extracts

On electrophoretical separation of the 1 M NaCl soluble myocardial AMPS at buffer pH 2 6 only 2 bands were seen again corresponding to fractions 1 and 2 in the crude MPS extract The results seem to indicate that in acidic buffer solution at pH up to 4 8 the myocardial fractions 2 and 3 had the same electrophoretic mobility

3 *Autoradiographic identification of ^{35}S containing MPS*

On autoradiography of electrophoretic strips containing MPS extracted with papain from the myocardium of the ^{35}S treated rats obvious blackening was seen on all autoradiographs at the site of fractions 2 and 3 (Fig 4) It was thus established that these fractions were sulphated and that fraction 1 was not sulphated which argues in favour of its being identical with HA

In this experiment fraction 3 was particularly strong and divided into two portions The ^{35}S activity was concentrated in the posterior portion of the band (Fig 4) After digestion with DNAase the anterior portion disappeared completely whereas the posterior ^{35}S-containing portion of the fraction remained unaltered The ^{35}S-containing portion on the other hand was completely digested by testicular hyaluronidase whereas the anterior portion was resistant to this enzyme The ^{35}S-

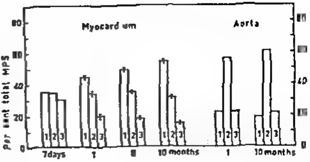

Fig 6 Relative proportions of the electrophoretically separated mucopolysaccharides from myocardium and aorta of normal rats of different ages Calculations based on chromoscanning Mean values ± S E M Electrophoretic details as in Fig 2 1—3 = Mucopolysaccharide fractions corresponding to these in Fig 2—5

containing portion therefore seems to be identical with that fraction 3 which was described in the foregoing As to the anterior portion of the band, it seems justifiable to assume that contamination with DNA had occurred in this experiment

The MPS pattern of myocardium and aorta at different ages

The myocardial MPS pattern was very much the same in all age groups (Fig 5 a—c) and differed markedly from the aortic curve (Fig 5 d) The HA peak was the dominant feature in the myocardial samples, while the fraction 2 peak was dominant in the aortic samples

The relative proportion of each MPS component was densitometrically determined in all myocardial and aortic extracts as a percentage of the total MPS (Fig 6) With increasing age a slight increase of HA can be noted in myocardium

Discussion

When MPS extracts from papain-digested myocardium and aorta were separated by electrophoresis three Alcian blue positive components were invariably demonstrated Fraction 1 in myocardium and aorta was considered to be identical with HA on the basis of its electrophoretic mobility its complete digestibility with testicular hyaluronidase and the absence of sulphation Fraction 2 in the myocardium was resistant to testicular hyaluronidase digestion and sulphated as revealed by autoradiography Although it was uncertain whether fraction 2 in the aorta was sulphated it may well be identical with fraction 2 in the myocardium considering their otherwise identical properties An AMPS component resembling the present factor 2 separated by electrophoresis from papain-digested human aorta by the same method as in the present study was considered as identical with HS (Manley 1965 Nakamura *et al* 1966) CS B and keratosulphate are sulphated AMPS with the same electrophoretic properties as HS (Nanto *et al* 1963) However, keratosulphate does not seem to occur in the aorta (Scharah *et al* 1968) A negligible proportion of the aortic chondroitin sulphates consists of CS B (Sirek *et al* 1964) and neither of these AMPS has been demonstrated in myocardium (Dalferes *et al* 1967) Fraction 2 may therefore be identical with HS which would mean that HS constitutes an important AMPS *in* both aorta and myocardium

Fraction 2 remained dissolved when myocardial MPS were precipitated with 0.5 % CPC in 1 M NaCl and it was not identified in the 2 N MgCl soluble fraction which seems to contain only highly sulphated AMPS (Scott 1960, Antonopoulos *et al.* 1964). Likar *et al.* (1968) investigated electrophoretically 0.5—2 M NaCl soluble fractions of mural AMPS from bovine coronary vessels eluted from Dowex 1 X2 columns. The 0.75 M fraction consisting chiefly of HS and the 1 M NaCl soluble fraction consisting of HS and CS A/C were weakly sulphated and migrated in pyridine acetate buffer (pH 3.5) as a single band between the HA and CS B references. The sulphated fractions 2 and 3 in the rat myocardium in the present study, like the 0.75 M and 1 M NaCl soluble bovine coronary fractions, migrated in acidic buffer (pH 2.6—3.6) as a single band between HA and CSB-HS. Considering what has been said above of its properties, fraction 2 in the rat myocardium may be identical with HS. However, this fraction contained no hyaluronidase sensitive component in contrast to the HS fraction described by Likar *et al.* (1968). This may be attributed to the fact that a more clear cut separation of AMPS is obtained by electrophoresis than by elution with rising concentrations from Dowex 1 X2 and cellulose columns (Manley and Kent 1963, Nanto *et al.* 1963).

Fraction 2 could not be definitely identified, however, and it could not be established whether it represented the same MPS component in the myocardium and aorta.

Fraction 3 in myocardium and aorta had the same electrophoretic mobility as the CS B reference at a buffer pH of 4.8—9.2. These substances were, however, digested testicular hyaluronidase and corresponded in this respect to CS A/C, while CS B entirely resistant to this enzyme. On electrophoresis in acidic buffer, aorta fraction 3 migrated to a site near the CS B reference, while myocardial fraction 3 migrated with fraction 2. Highly sulphated AMPS, like the chondroitin sulphates, maintain their electrophoretic mobility even in highly acidic buffer solution, while the mobility of weakly sulphated AMPS containing a small number of dissociable ester sulphate groups, or HA which only dissociates into free carboxyl groups, is reduced with decreasing buffer pH (Foster and Pearce 1961). The difference in electrophoretic mobility in acidic buffer as compared to the corresponding myocardial fraction 3 may thus be due to a higher degree of sulphation in aortic fraction 3, as indicated above (Foster and Pearce 1961, Mathews and Decker 1968). Similarly, the fact that myocardial fractions 2 and 3 had the same mobility in acidic buffer solution may be due to their being weakly sulphated AMPS.

Summing up, fraction 3 in the myocardium seems to be a CS A/C-like, probably weakly sulphated AMPS with a molecular structure not corresponding to that of aortic fraction 3. A corresponding fraction from normal human aorta isolated and electrophoretically separated by identical methods by Manl[illegible] (196[illegible]) and Nakamura *et al.* (1966) showed the same property as aortic fr[illegible] 3 in the present study and was considered to be CS A/C.

Exact identification of electrophoretically [illegible]d MPS [illegible] ever. The chondroitin sulphates contain g[illegible] ine [illegible]

keratosulphate which contain glucosamine Gas chromatographic analysis of the glucosamine and galactosamine content in pooled AMPS bands from electrophoretic strips would therefore be a more exact method for the identification of small amounts of AMPS (Lehtonen *et al* 1966)

Chromoscanning of the electrophoretic strips revealed characteristic MPS patterns in myocardium and aorta respectively The HA fraction was predominant in myocardium and fraction 2 was found to constitute the main proportion of the MPS content in aorta and the next largest component in myocardium

Berensen *et al* (1968) isolated AMPS from human myocardial tissue and fractionated them on Dowex 1 X2 columns HA and HS were found to be the predominant AMPS Dalferes *et al* (1967) showed that the HA fraction constituted 60 per cent and HS about 30 per cent of the total AMPS content in the normal human myocardium while CS-A/C constituted 10 per cent CS B on the other hand could not be demonstrated This is in agreement with the MPS pattern in normal rat myocardium observed in the present study provided that fractions 1 2 and 3 correspond to HA HS and CS A/C respectively

On densitometric estimation of the relative proportions of electrophoretically separated MPS several factors must be taken into account According to Manley (1965) the Alcian blue binding capacity of HA is approximately half that of chondroitin sulphate on a weight for weight basis On chromoscanning a too low relative amount of HA is therefore obtained Different degrees of sulphation of the chondroitin sulphates possibly influence their Alcian blue binding capacity and thus also the results of densitometric estimation of chromoscanning (Manley 1965)

Hence if fraction 3 in the myocardium is a CS-A/C like MPS with a low degree of sulphation it ought to bind a smaller amount of Alcian blue than the corresponding aortic fraction In addition, the peak of fraction 2 was not completely separated from the HA peak in the chromoscans However since the variations in the proportions of the myocardial MPS fractions in the different age groups were small the following conclusions may be drawn

The proportions of the MPS components in the rat myocardium corresponded well to the composition of AMPS in human myocardium as fractionated from Dowex 1 X2 columns (Dalferes *et al* 1967) In contrast to the results obtained on aorta and heart valves a slight increase of the HA fraction and a corresponding decrease of the CS-A/C fraction were observed in the rat myocardium with increasing age The HS-like fraction 2 showed the smallest relative change Investigations on the AMPS composition in human aorta Manley 1965 Kumar and Berenson 1967) also suggest that the amount of HS remains fairly constant with increasing age

No age related decrease in the content of MPS in dry myocardial tissue or MPS extract from papain-digested myocardium could be demonstrated by hexosamine determination or in alkali-extracted myocardial tissue by uronic acid determination. The hexosamine concentration in a protein free MPS concentrate from papain digested rat myocardium was 0 9—1 μg/ml dry weight (v Knorring 1970 b) which corresponds to the hexosamine content in the crude MPS extract used in the pre-

investigation This low amount of hexosamine was indicative of a low amount of neutral MPS and other "non AMPS" hexosamine-containing substances in the crude MPS extract in question The concentration of uronic acid in alkali extracted rat myocardium was low and varied from 0 21—0 47 µg/mg dry weight Similar results were obtained on protein free MPS concentrates of papain digested rat myocardium (v Knorring 1969 b) Dalferes *et al* (1967) indicated the amount of carbazol uronic acid in normal human myocardial tissue at 0 42—0 52 µg/ml dry weight, which is in agreement with the figures for uronic acid in the rat myocardium obtained in this study

In conclusion just as the content of collagen (v Knorring 1970 a), the content of MPS in the rat myocardium remained more or less constant with increasing age The changes in the myocardial MPS pattern observed were slight and seemed rather to be in contrast to the age changes reported in the MPS pattern in aorta and heart valves The development of the connective tissue framework of the myocardium seems to be completed at an early foetal stage and in normal cases undergoes only insignificant age changes

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References

Antonopoulos C A S Gardell J A Szirmai and E R de Tyssonsk Determination of glycosaminoglycans (mucopolysaccharides) from tissues of the microgram scale *Biochim biophys Acta* (Amst) 1964 *83* 1—19

Berenson G S B Radhakrishnamurthy and E R Dalferes Acid mucopolysaccharide changes in amyloidosis of the heart *Fed Proc* 1968 *27* 413

Bertelsen S Hexosamine hydroxyproline and calcium levels in the intima of the human aorta as related to age and atherosclerotic changes *J Geront* 1962 *17* 24—26

Boas N F Method for the determination of hexosamine in tissue *J biol Chem* 1953 *204* 553—563

Bollet A J D V Anderson and W F Simpson The measurement of tissue acid mucopolysaccharides *J clin Invest* 1958 *37* 858—863

Bostrom H A Moretti and M Whitehouse Studies on the biochemistry of heart valves I On the biosynthesis in bovine heart valves *Biochim biophys Acta* (Amst) 1963 *74* 213—221

Bottcher C J and F B Kleinstra Content of acid mucopolysaccharides in the human aorta *J Atheroscler Res* 1962 *2* 263—269

Clausen B Influence of age on connective tissue uronic acid and uronic acid hydroxyproline ratio in human aorta myocardium and skin *Lab Invest* 1962 *11* 1340 1345

Dalferes E R Jr B Radhakrishnamurthy and G S Berenson Acid mucopolysaccharides of amyloid tissue *Arch Biochem* 1967 *118* 284—291

Dische Z A new specific color reaction for hexuronic acids *J biol Chem* 1947 *167* 189—198

Elson L A and W T Morgan A colorimetric method for the determination of glucosamine
1826
sis of acid mucopolysaccharides *Canad J*

The identification of a sulfated mucopoly
nat Acad Sci (Wash) 1963 *49* 643—
647

Gessner I H and H Bostrom In vitro studies of ³⁵S sulfate incorporation into the acid mucopolysaccharides of chick embryo cardiac jelly *J exp Zool* 1965 *160* 283—290

Hauss W H and G Junge Hulsing Uber die universelle unspezifische Mesenchymreaktion *Dtsch med Wschr* 1961 *86* 763 768

KAPLAN, D and K MEYER, Mucopolysaccharides of aorta at various ages *Proc Soc exp Biol* (N Y) 1960 *105* 78—81
KIRK, J E and M. D DYRBYE, Mucopolysaccharides of human arterial tissue I Isolation

1967 *7* 573—582

leic acid (desoxy-

with ^{35}S-sulphate
hop scand 1967.
Suppl 106
LEHTONEN, A, J KÄRKKÄINEN and E HAAHTI, Gas chromatographic characterization of the electrophoretically separated fraction of acid mucopolysaccharides *J Chromatogr* 1966 *24* 179—182
LIKAR, I N, L J LIKAR and R. W ROBINSON, Levels and patterns of acid mucopolysaccharides in bovine coronary arteries without gross lesions *Acta endocr.* (Kbh) 1968 *59* 668—681
LOEWI, G and K MEYER, The acid mucopolysaccharides of embryonic skin *Biochim biophys Acta* (Amst) 1958 *27* 453—456
LOWTHER, D A, B P TOOLE and F A MEYER, Extraction of acid mucopolysaccharides from bovine heart valves *Arch Biochem* 1967 *118* 1—11
MANLEY, G W and P W KENT, Aortic mucopolysaccharides and metachromasia in dissecting aneurysm *Brit J exp Path* 1963 *44* 635—640
MANLEY, G, Changes in vascular mucopolysaccharides with age and blood pressure *Brit J exp Path* 1965 *46* 125—134
MATHEWS, M B and L DECKER, The distribution of sulfate groups in chondroitin sulfate chains Detection of "oversulfated" and "undersulfated" repeating periods *Biochim biophys Acta* (Amst) 1968 *156* 419—421
NAKAMURA, N, Y ISHIHARA, T SATA and N YABUTA, Acid mucopolysaccharides and lipids of Japanese arteries with special references to the absence of hyaluronic acid in Japanese cerebral artery *J Atheroscler Res* 1966 *6* 132—150
NANTO, V, On the electrophoretic separation of acid mucopolysaccharides on cellulose acetate sheets *Acta chem scand* 1963 *17* 857
SCHARAH, C J, D LORELL and R C CURRAN, Arterial acid mucopolysaccharide concentrations, their correlation with age and intimal hyperplasia *Brit J exp Path* 1968 *44* 574—585
SCHILLER, S and A DORFMAN, Effect of age on the heparin content of rat skin *Nature* (Lond) 1960 *185* 111—112
SCOTT, J E, *Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues* In Methods of Biochemical Analysis Vol 8, Ed D Glick, Interscience Publishers, New York 1960 145—197
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From the Institute of Biological Chemistry, University of Copenhagen, Denmark

The Effect of Amiloride on Sodium Transport in the Normal and Moulting Frog Skin

By

ROBERT NIELSEN AND R W S TOMLINSON[1]

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Abstract

NIELSEN, R and R W S TOMLINSON *The effect of amiloride on sodium transport in the normal and moulting frog skin* Acta physiol scand 1970 79 238—243

It is shown that amiloride is a potent inhibitor for the active sodium transport across the isolated frog skin The percentage inhibition produced by various amiloride concentrations was not dependent on the initial short-circuit current but rather on the phase of the moulting cycle at which the skin was tested

The pyrazine diuretic 3 5 diamino 6 chloropyrazinoylguanidine (amiloride) has been shown to reduce the sodium transport across the renal tubule (Baer *et al* 1967), toad bladder (Bently 1968) and frog skin (Baba *et al* 1968) Its action on the toad bladder is attributed to a restriction of the entry of sodium across the mucosal side of the toad bladder epithelial cells It seemed of interest to investigate the action of amiloride on another widely used transporting epithelium namely the frog skin It is shown that amiloride is also a potent inhibitor for the active sodium transport across the isolated frog skin Furthermore it is shown that the percentage inhibition produced by various amiloride concentrations depends on the stage in the moulting cycle at which the skin was tested

Methods

The experiments were performed on the ventral abdominal skins of *Rana temporaria* The animals had been stored in a cold room at 4° C prior to use

The frogs were doubled pithed and the skins dissected and rinsed in Ringer's solution between two glass chambers and the potential and short circuit current measured according to Ussing and Zerahn (1951) and recorded automatically

The sodium efflux was measured using Na^{22} (Amersham England) The first samples were withdrawn after a 20 minute equilibration period and one hour thereafter On removing the amiloride containing outside Ringer's solution and replacing with fresh Ringer's solution

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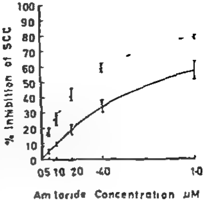

Fig 1 Percentage inhibition of the short-circuit current with increasing concentrations of amiloride in the outside bathing solution Broken line normal skins Continuous line skins in which it was known that shedding of the stratum corneum had taken place 1 1/2—3 1/2 hrs previously Error bars are ±S E of 12 skins (broken line) or 4 skins (continuous line)

pH was 7 8—8 0

The amiloride (MK 870) was a gift from Merck Sharp and Dohme Ltd The aldosterone used was "Aldocorten" (Ciba Ltd)

Results

Short circuit current (scc) It is well established that the short-circuit current of the frog skin is a measure of the active sodium transport (Ussing and Zerahn 1951) Figure 1 shows the relationship between the percentage inhibition of the scc and amiloride concentration on the outside bathing fluid on twelve randomly chosen frog skins An amiloride concentration as low as 5×10^{-7} M produces within 30 sec a reduction in the scc and at a concentration of 2.5×10^{-6} M a 90 % inhibition is obtained There is also a rapid decline in the potential difference but not of the same magnitude as the scc so that there is an increase in resistance The skins were exposed to the various amiloride concentrations for two minutes and on removing the amiloride solution and rinsing once both the scc and the potential difference returned immediately to their normal values Similar concentrations of amiloride applied to the inside bathing solution were without any effect

The percentage inhibition produced by various amiloride concentrations was not dependent on the initial short circuit current but rather on the phase of the moulting cycle at which the skin was tested This moulting cycle *in vitro* has recently been discussed by Nielsen (1969) and is characterised electrically by a gradual decrease to very low levels of both the potential and the short circuit current (the inhibition period) followed by a gradual increase in the short circuit current and potential (the activation period) During the latter part of the inhibition period the stratum corneum is detached from the underlying cells and is easily removed The duration of this moulting process is approximately eight hours During this series of experiments four skins moulted spontaneously while they were mounted between the

TABLE I Effect of 5×10 M am lor de on the sod um efflux

	Sod um efflux μ eq/hr/cm²		
Exp No	Control	Am lor de	Control
1	0 067	0 072	0 076
2	0 087	0 092	0 109
3	0 035	0 042	0 048
4	0 050	0 053	0 086
5	0 055	0 059	0 065

chambers and an amiloride dose response curve was performed on them 1 1/2 to 3 1/3 hrs after the separation of the stratum corneum from its underlying cells The result is shown in Fig 1 where it is apparent that there is a diminished inhibition in these skins compared to the twelve randomly selected skins tested previously

Sodium efflux Sodium efflux measurements were performed on five skins for one hour period before during and after the addition of 5×10⁻⁷ M amloride to the outside bathing fluid The results are shown in Table I This concentration of amloride produced only small changes in the sodium efflux These changes cannot account for the inhibition of the short circuit current and it must therefore be concluded that the principal effect of amiloride is on the influx of sodium

Aldosterone treated skins It has been shown by Nielsen (1969) that incubating

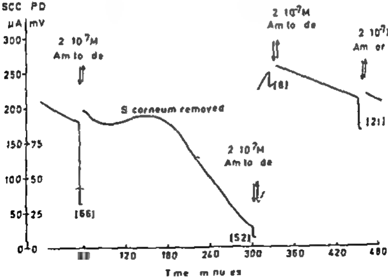

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TABLE II The percentage inhibition of the scc by amiloride before and after the onset of the aldosterone induced inhibition

% inhibition of scc		
Amiloride 2×10^{-7}M before	Aldosterone	Amiloride 2×10^{-7} after
53 0	57 0	56.5
61 5	55 5	68 5
66 0	87 0	52 0
52 5	63 0	63 0
62 0	53 5	71 0
66 0	82 0	52 0
56 0	74 0	59 0
60 0	69 0	57 5

skins of *R temporaria* with aldosterone induces a moult *in vitro*, and it was decided to try the effect of amiloride at different stages of an aldosterone treated skin. Fig 2 shows a typical result from 12 such experiments It will be seen that amiloride produces a good inhibition during the early part of the experiment and even just before the removal of the stratum corneum On removing the stratum corneum however, there is a diminished or no inhibition of the scc with the same dose of amiloride and to achieve the same degree of inhibition obtained previously it is neces-

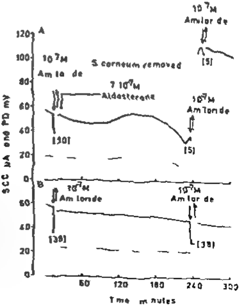

Fig 3 Short-circuit current (solid line) and potential difference (dotted line) in two skin halves from the same frog Amiloride was added to the outside bathing fluid and after three minutes removed by changing the Ringer's solution.

A — Aldosterone treated skin half
B — Control skin half

Figures in parentheses are the percentage inhibition produced by the amiloride

sary to use higher doses (×10 or greater). The skin's ability to respond to amiloride is gradually restored with time.

The percentage inhibition produced by amiloride just before the removal of the stratum corneum is variable to the extent that the time chosen for the addition of the amiloride is critical. If the amiloride is added when the scc is obviously still in the inhibition period nearly the same percentage inhibition is obtained as before the onset of the aldosterone induced inhibition (Table II). If the amiloride, however, is added when the scc is just increasing after the inhibition period, then there is no or a very low inhibition (Fig. 3). Control experiments were performed in which six skins without aldosterone were treated with increasing concentrations of amiloride before and after the corneum has been rubbed but of course not removed. There was no significant difference in their response to amiloride after this manual treatment.

Discussion

Bentley (1968) showed that amiloride was a powerful inhibitor of sodium transport in the toad bladder. The present experiments show that the frog skin is just as sensitive and that the sensitivity is dependent upon the stage of the skin in its moulting cycle when the tests are performed.

The inhibition of the short circuit current is a direct effect on the sodium influx as the efflux has been shown to be only slightly affected during the amiloride treatment.

It is apparent from Fig. 1 that skins which have just undergone a moult a few hours before the addition of the amiloride exhibit a diminished response, which suggests that in a normal skin one of the sites of amiloride action must be located at the outermost border of the skin. The outermost border can be divided into three parts: 1) the outer cell membrane of the stratum granulosum, 2) the material between the stratum corneum and the stratum granulosum, 3) the stratum corneum.

The experiments with aldosterone treated skins were performed to test this possibility. Nielsen (1969) has shown that aldosterone in vitro induces a moulting cycle in frog skin, i.e. a separation of the stratum corneum from the underlying cells. Skins which exhibited an initial good response to amiloride show also a good response during the inhibition phase of the moulting cycle (Table II), whereas the inhibition was nearly abolished when the skin was in the activation period or if the stratum corneum was removed. Thus amiloride still produces a good inhibition when the skin is in the latter part of the inhibition period although the material between stratum corneum and the stratum granulosum is broken down (the corneum is easily removed at that time). This indicates that the material between the stratum corneum and the stratum granulosum is probably not the site of action. It has been suggested that the onset of the activation period during the moult is accompanied by a breakdown of the corneum (Nielsen, 1969). If it is so, then it must be most likely that the action of amiloride is caused by a blocking of sodium channels in the corneum. This would also explain why the inhibition is nearly abolished when the corneum is re-

moved The ability to respond to amiloride some hours after the removal of the corneum must then be due to the formation of a new corneum The fact that amiloride can produce a small inhibition after the removal of the corneum indicates that it also has another site of action in the frog skin This site is probably the outer cell membrane of the stratum granulosum

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References

BABA W I, A F LANT, A J SMITH M M TOWNSHEND and G M WILSON Pharmacological effects in animals and normal human subjects of the diuretic amiloride hydrochloride (M K 870) *Clin Pharmacol Ther* 1968 9 318—327

BAER J E C … sium sparing and natriuretic activity of … hydrochloride (amiloride hydrochlorid …

BENTLY, P J … rt across the toad bladder *J Physiol* (Lond) 1968 *195* 317—330

NIELSEN R The effect of aldosterone in vitro on the active sodium transport and moulting of frog skin *Acta physiol scand* 1969 *77* 85—94

USSING H H and K ZERAHN Active transport of sodium as the source of electric current in the short-circuited isolated frog skin *Acta physiol scand* 1951 *23* 110—127

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Metabolic Actions in Intestinal Smooth Muscle Associated with Relaxation Mediated by Adrenergic α- and β-Receptors

By

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Received 30 September 1969

Abstract

Andersson, R and E Mohme Lundholm *Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α- and β receptors* Acta physiol scand 1970 79 244—261

In colonic muscle from the rabbit a relaxing effect was induced by stimulation both of adrenergic α receptors with phenylephrine and of β receptors with isoprenaline The α receptor induced relaxation was not accompanied initially by any metabolic effects After a latency period of 3 min did a decrease in the phosphorylase *a* activity, a decrease in the concentrations of hexose phosphates and lactate and an increase in the concentration of high energy phosphate compounds (ATP and CrP) become evident There was a decrease of the cyclic AMP content These effects were inhibited by dibenamine The relaxation induced via adrenergic β receptors was preceded by an increase in the cyclic AMP content and the phosphorylase *a* activity, an increase in the concentration of hexose phosphates and lactate [illegible]

muscle The relaxing and metabolic effects of isoprenaline could be [illegible] cyclic AMP only incompletely by 5 AMP and not at all by other tested cyclic nucleotides

Ahlquist and Levy (1959) showed that a relaxing effect in intestinal muscle was mediated via both adrenergic α and β receptors We found that the relaxation was induced by means of different mechanisms when α and β receptors were stimulated by l phenylephrine and l isoprenaline respectively (Andersson and Mohme-Lundholm 1969 a) The relaxation induced via α-receptors was inhibited by factors *which tended to even out the ionic gradients of* K and/or Na across the cell membrane The relaxation mediated by β receptors was potentiated by theophylline and puromycin and blocked in a carbohydrate poor' muscle and was assumed to result from a metabolic effect probably induced by an increased formation of cyclic AMP

The relation between the relaxing and metabolic effects of the catecholamines in smooth muscle has been the subject of a number of investigations, which have given partly controversial results (review Lundholm Mohme-Lundholm and Svedmyr 1966)

In the present investigation we have studied the metabolic responses associated with relaxation produced by selective stimulation of adrenergic α- and β receptors, respectively In previous investigations the effects of the catecholamines on the carbohydrate metabolism of smooth muscle and the metabolism of high energy phosphate compounds were mainly studied, and we have therefore concentrated primarily on these effects Our results indicate that the divergent opinions on the relation between the relaxing and metabolic effects of the catecholamins can be explained by the fact that on stimulation of adrenergic α- and β receptors in smooth muscle completely different metabolic effects are obtained A preliminary report of the results of these experiments has been published (Andersson and Mohme Lundholm 1968)

Methods

The experiments were performed on colonic muscle from the rabbit After killing the animal by a blow on the neck the colon
Henseleit bicarbonate buffer at 20
layer of muscle From the latter,
long and weighing 0 13—0 18 g
the longitudinal direction of the
1966) The distance between the
and the tension of the specimen
polygraph The specimen and holder were then immersed in an organ bath containing 30 ml Krebs-Henseleit bicarbonate buffer with a glucose content of $11\ 5\times10^{-3}$ M at 37° C The suspension solution was bubbled with 95 % O_2+5 % CO_2

The specimens were then stretched successively until they exerted a constant tension of 1 g after which they were kept in the organ bath for 60 min During this time the ATP and CrP concentrations in the muscle increased The suspension solution was then changed for glucose free solution so that variations in the glucose uptake should not influence the concentration of hexose phosphates

... muscle specimens were contracted with K ions the catecholamines were added 10 min after the potassium ions

In the experiments with cyclic AMP 5 AMP or other cyclic nucleotides these compounds were added 10 min after carbamylcholine and the specimens were frozen after 60 sec In the experiments with dinitrophenol the specimens were frozen 5 min after the addition of the compound

Generally 4 specimens were mounted at the same time One to two of these were controls and were only contracted with carbamylcholine or K ions while catecholamines or nucleotides were also added to the other two In the experiments in which the effects of adrenergic block
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used In the experiments on carbohydrate poor muscle the specimens were first incubated for 90 min in glucose free Krebs-Henseleit bicarbonate solution bubbled with 100 % N_2 The suspension solution was then changed for a Krebs Henseleit solution in which the glucose was replaced by 11 5 mM Na pyruvate (pH 7 40) The solution was aerated with 95 % O_2+ 5 % CO_2 About 5 min later carbamylcholine was added and after a further 10 min isoprenaline Since lactic acid can be expected to disappear from the muscle rapidly by diffusion the experiments in which the lactate formation was determined were carried out in a humid chamber

The specimens were first suspended in Krebs Henseleit bicarbonate buffer for 60 min and were then hung up in a humid chamber filled with 95 % O_2+5 % CO_2 at 37° C Glucose free Krebs Henseleit bicarbonate buffer saturated with the same gas mixture was poured over the specimens from an infusion apparatus at a constant rate of 0 3 ml/min Carbamylcholine and catecholamine derivatives were added to the solution The specimens were then frozen and treated in the usual way

The specimens were frozen at −80°
phosphorylase activity was determined
0° C in a solution containing 0 02 M
and 0 3 % serum albumin The phos
curring reaction direction according to the method of Bueding *et al* (1962), the amount of G 1 P formed in the reaction glycogen+H_3PO_4 $\xrightarrow{\text{phosphorylase}}$ G 1 P, being determined enzymatically The reaction was carried out both in the presence of 0 001 M AMP (=total phosphorylase activity) and in the absence of AMP (=phosphorylase *a* activity) The phos
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The values for the enzyme activities determined according to Bueding *et al* (1962) were somewhat lower (total phosphorylase activity 1.8±0 3 µmoles G 1 P/g tissue/min 6 6±1 1 % phosphorylase *a* activity) and showed less variation than in the method of Cori *et al* (total activity 2 2±0 3 µmoles P_i/g/min, 12 4±3 8 % *a* activity) Since the method of Bueding *et al* showed lower and more even values of the phosphorlyase *a* activity it was considered preferable in these experiments

The remaining part of the muscle was homogenized at 0° C in 8 vol 6 % perchloric acid After neutralization with K
6 phospate (G 6 P fructo
adenosine triphosphate (AT
adenosine monophosphate
determined according to Be
F 1 6 P according to Buche
Stein (1963) ADP and AM
me Lundholm and Var
Rall (1970

Results

Adrenergic α and β receptors in colonic muscle from the rabbit

In order to obtain sufficient amounts of tissue for determination of the metabolite concentration the muscle layer from the rabbit colon was used for the experiments instead of rabbit taenia coli as used previously (Andersson and Mohme Lundholm 1969 a)

In the first series of experiments a study was made of the concentrations of l phenylephrine and l isoprenaline that were required in the rabbit colon for selective stimulation of adrenergic *α* receptors and of adrenergic *β* receptors The treshold concentration for the relaxing effect of isoprenaline was found to be higher (4×10^{-9} g/ml free base) in colonic muscle than in taenia coli from the rabbit ($=4\times10^{-8}$ g/ml) Sotalol ($1\ 2\times10^{-5}$ g/ml) blocked completely the effect of isoprenaline in a concentration of 5×10^{-7} g/ml The effect of l phenylephrine ($\leq1\ 1\times10^{-6}$ g/ml) was completely blocked by dibenamine in a concentration of 5×10^{-6} g/ml Sotalol in that concentration had no influence on the effect of phenylephrine Within this given range of concentration phenylephrine and isoprenaline induced relaxation via selective adrenergic *α* -or *β* receptor stimulation The concentration of ATP in the colonic muscle showed seasonal variations with a maximum

TABLE I Influence of l isoproterenol (5×10^{-7} g/ml) and l phenylephrine (1.1×10^{-6} g/ml) at activity in percent of total phosphyrolase activity, content of hexose phosphates, ATP, significance of the effect is denoted by * = P<0 05, ** = P<0 01, *** = P<0 001

Drugs	n	Relax per cent	Phosphorylase *a* per cent
Control values		—	7 7±0 7
ΔIsoprenaline, 30 sec	16	2 5±2 0	+2 1±0 8*
Control values		—	6 9±1 1
ΔIsoprenaline, 60 sec	8	38±3 0***	+8 6±2 1**
Control values		—	9 0±0 6
ΔIsoprenaline, 90 sec	8	40±3 0***	+8 6±2 1**
Per cent of basal values			195
Control values			4 9±0 5
ΔIsoprenaline, 10 min	9	36±3 0***	+1 3±0 2***
Control values		—	9 0±0 6
ΔPhenylephrine, 45 sec	10	88±2 5***	+0 3±0 9
Control values			7 3±0 6
ΔPhenylephrine, 3 min	6	79±5 0***	—1 6±0 6*

during October—February and a minimum during May—August which finding explains the marked variations in the basal ATP concentration (Table I—III) The nature of the observed effects of catecholamines was the same, however, at different basal ATP concentrations which was shown by control studies carried out in all series of experiments with catecholamines

Metabolic effects associated with adrenergic α receptor stimulation

In colonic muscle contracted with carbamylcholine l phenylephrine (1.1×10^{-6} g/ml) induced a relaxing effect which was quickly manifested and reached its

TABLE II Influence of sotalol (1.2×10^{-5} g/ml) on the relaxing and metabolic effects of isoprenaline of dibenamine (5×10^{-6} g/ml) on the actions of phenylephrine (1.1×10^{-6} g/ml) in

	Drugs	n	Relax per cent	Phosphorylase *a* per cent
	Control values	8	—	6 9±1 1
1 Δ	Isoprenaline 60 sec	8	42±6***	+2 5±0 7*
2 Δ	Sotalol	8	19±1***	—0 2±1 0
3 Δ	Sotalol—(Sotalol+Isoprenaline)		—4±2	—0 5 0 8
Δ	1—3		38±6***	+3 0±1 0*
	Control values	7	—	5 6±0 5
4 Δ	Phenylephrine 3 min	7	80±3***	—2 3±0 5**
5 Δ	Dibenamine	7	25±1***	—2 5 0 6**
6 Δ	Dibenamine—(Dibenamine+Phenylephrine)	7		+0 9 0 7
Δ	4—6	7		—3 2±0 9**
	Control values	7	—	5 8 1 3
Δ	DNP, 5 min	7	32±3 5	+4 7 0 9**
Δ	Sotalol	6	19±1	—1 6 0 8
Δ	Sotalol—(Sotalol+DNP)		—	—6 9±1 6**

different times on, relaxation (decrease of tension in per cent of initial tension), phosphorylase *a* CrP in nmole/g and lactate in µmole/g wet weight of rabbit colon Δ = change from control values Statistical n = no of tests

G-1-P	G 6-P	F-6-P	F-1-6-P	ATP	CrP	Lactate
16±2	23±3	7±1	57±14	538±37	171±11	3 8±0 5
+6±4	+2±2	+6±2**	+9±5	−120±43*	−46±14**	0 9±0 3*
14±3	18±4	8±1	33±6	449±65	131±24	—
+14±6*	+14±8	+4±1**	+12±4*	−124±38*	−31±10*	—
20±3	19±3	6±1	28±9	521±62	188±14	5 3±1 6
−15±6*	+15±8	+6±2*	+10±8	−274±89**	−101±38*	+0 9±0 2**
175	179	200	137	42	46	117
18±2	33±5	13±1	24±6	114±21	45±5	—
+6±2*	+7±2*	+5±2*	+10±2*	+40±17**	+17±17**	—
20±3	19±3	6±1	28±9	524±62	188±14	3 8±0 7
−3±3	+2±1	+1±1	−9±4	+15±59	+28±26	−0 7±0 2**
28±5	53±6	14±2	40±2	101±11	35±6	—
−20±3**	−28±8*	−6±2*	−20±4**	+91±14**	+20±4**	—

maximum within only 20 sec when the tension decreased from approximately [illegible] to 1 p (Fig 1) There was after 60 sec a reduction of the cyclic AMP content which fell with $-0.77 \pm 0.37 \times 10^{-10}$ mole/g from a basal value of $2.6 \pm 0.29 \times 10^{-10}$, ($n = 7$, $P = 0.05$) *When the effect on the phosphorylase activity and the metabolite concentrations in the muscle were determined* 45 sec after the addition of phenylephrine no significant changes were observed apart from a lactate reduction and a tendency to a decrease in the concentration of fructose-1-6 diphosphate (Table I) [A]fter 3 min however both a significant decrease of the phosphorylase *a* activity and [a] decrease of the concentrations of different hexose phosphates had occurred The [co]ncentrations of high energy phosphate compounds (ATP and CrP) had increased significantly (Table I) Dibenamine (5×10^{-6} g/ml) had in itself a relaxing action

(5×10^{-7} g ml) and the corresponding actions of dinitrophenol DNP (6.6×10^{-5} M) Influence rabbit colon For symbols see Table I

G 1 P	G 6 P	F 6 P	F 1 6 P	ATP	CrP
14±3	18±4	8±1	33±6	449±65	131±24
+14±6	14±8	−4±1*	+12±4*	−124±38*	31±10*
−2±2	3±2	2±1	+12±6	−84±17***	16±8
−1±4	11±3*	0±1	6±6	+77±37	−3±8
+15±8	25±9*	+4±1*	+17±7*	−201±53**	−28±13*
22±4	80±12	18±2		85±6	54±[illegible]
−11±2**	40±11*	−6±2		+13±10	−7±8
−8±3*	−34±9*	−4±2	—	+1±6	+1±9
−3±2	+10±8	2±1		+3±8	−17±10
−8±3*	50±13**	[illegible]±3*		+10±13	+10±14
28±6	32±9	16±5	31±8	261±49	129±18
22±8*	+14±9	24±11	+12±10	−125±30**	−63±11*
+1±6	+9±6	+3±4	+1±10	−47±40	−13±28
+9±9	−8±6	+3±2	+26±11*	−74±14**	−55±20*

[illegible] rabbit colon of isoproterenol [illegible]

	Drugs	n	Relax per cent	Phosphorylase *a* per cent
A	Control values	4	—	4 6±0 7
1 Δ	Isoproterenol	7	31±3***	+5 2±1 4**
B.	'Carbohydrate poor'	4	—	2 7±0 4**
2 Δ	Isoproterenol	8	±0±0	+0 1±0 4
Δ	1—2	—	31±3***	+5 1±1 5**
C.	Control values	5	—	11 8±1 9
Δ	145 meq K+	5	—	+6 5±2 1*
Δ	Iso 145 meq K	10	23±3***	+5 9±1 9
D	Control values	5	—	4±0 9
Δ	D butyryl cyclic AMP	5	±0±0	±0±0 2
E	Control values	9	—	7 9±2 1
Δ	Cyclic AMP	9	47±2***	+5 4±1 2**
Δ	5 AMP	6	32±6**	—0 4±3 7

and decreased the phosphorylase *a* activity and the concentrations of hexose phosphates Both the relaxing effect of penylephrine and its metabolic effects were blocked by dibenamine (Table II)

Metabolic effects associated with adrenergic β receptor stimulation Time response curve after isoprenaline treatment

Isoprenaline in a concentration of 5×10^{-7} g/ml induced a relaxation which became manifest considerably later than after treatment with phenylephrine and had a latency period of about 20 sec Our results are in agreement with the results of Brody and Diamond (1967) The relaxation did not reach its maximum until after 50—60 sec and was less complete than after the *α* receptor stimulation, the tension decreasing from about 8 to 5 p, or by about 40 % (Fig 1)

Already after 10 sec there was a significant increase in the cyclic AMP content with $1\,83\pm0\,38\times10^{-10}$ mole/g from a basal value of $5\,14\pm0\,87\times10^{-10}$, (n=5, P<0 02) A significant increase of the phosphorylase *a* activity took place after 20 sec *i e* before a significant relaxation was observed There was no change of the total phosphorylase activity Of the hexose phosphates F 6 P increased significantly, as also did the lactate concentration The concentrations of ATP and CrP decreased significantly (Fig 2) After 60 and 90 sec when the relaxation had reached its maximum the effect on the metabolite concentrations was stronger The phosphorylase a activity had increased further after 90 sec and both the G 1 P and F 6-P concentrations were significantly increased after both 60 and 90 sec After 60 sec the concentrations of fructose 1 6 diphosphate was also significantly increased and the lactate concentration was still elevated after 90 sec After 90 sec the concentrations of ATP and CrP had also decreased further (Table II)

(5×10⁻⁷ g/ml, 60 sec) on A normal, B "carbohydrate poor" preparation, C isoproterenol (5× AMP (1 1×10⁻⁶ mole/ml, 180 sec), E cyclic AMP (1 1×10⁻⁶ mole/ml, E 5′ AMP (1 1×10⁻⁶

G 1 P	G-6-P	F 6 P	F-1 6-P	ATP	CrP
27±4	47±9	12±2	—	135±14	56±11
+18±5**	+21±6*	+4±3	—	−49±14**	−29±11*
6±2*	6±1**	7±1**	—	53±11*	65±7
−1±1	+1±1	−1±5	—	7±6	±0±2
+19±5**	+20±5**	+5±6		−56±15**	−29±11*
26±4	57±9	21±5	37±6	61±10	36±9
+11±6	−12±17	−4±9	+2±3	+3±9	+1±7
−7±4	+13±8	+4±3	+15±4**	−18±3***	−22±4**
25±1	42±1	13±2	22±11	96±5	44±4
−6±3	−10±3*	−1±1	−9±4	+5±3	±5±5
27±7	57±3	18±5	19±5	194±13	88±24
+14±4**	−4±7	+3±4	+8±2**	−77±17**	−35±12*
−4±2	−5±3	−4±3	+12±7	−43±16*	+6±25

If the effect of isoprenaline on the metabolite concentration was determined after 10 min, when the relaxation was unchanged an increase in the phosphorylase *a* activity and the concentration of hexose phosphate persisted, but the ATP and CrP concentrations had increased significantly (Fig 2)

Changes in the metabolite concentrations after isoprenaline treatment

able I shows the percentual changes of different metabolites 90 sec after treatment ·h isoprenaline when the effects had reached their maximal strength The changes of the phosphorylase *a* activity and the concentrations of G-1-P, G-6-P and F-6-P

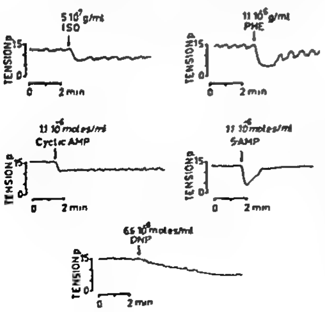

Fig 1 The muscle layer from rat but colon suspended in Krebs-Henseleit bicarbonate buffer without glucose and bubbled with 95 % O_2+5 % CO_2 at 37° C Recording of change in tension of the circular muscle layer The tension was increased initially by carbamylcholine 2 5×10⁻⁷ g/ml Relaxing effects of (A) Isoprenaline (ISO 5×10⁻⁷ g/ml free base), (B) Phenylephrine PHE 1 1×10⁻⁶ g/ml free base) C Cyclic AMP 1 1×10⁻⁶ mole/ml (D) 5 AMP 1 1×10⁻⁶ g/ml and (E) dinitrophenol DNP 6 6×10⁻⁶ mole/ml)

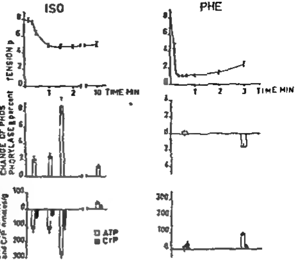

Fig 2 Time-response curves for l-isoproterenol (5×10^{-7} g/ml, ISO) and l-phenylephrine (1.1×10^{-6} g/ml, PHE) Effect on tension and change of phosphorylase *a* activity, ATP and CrP in relation to basal values Mean ± S.E.M. (n=7)

were of equal magnitude and amounted to 175—200 % of the basal values On the other hand, the change of F-1-6-P amounted to only 137 % In vascular smooth muscle (Beviz and Mohme Lundholm 1965) and skeletal muscle (Beviz, Mohme-Lundholm and Svedmyr 1967) a proportionally stronger increase in the concentration of F-1 6-P than of other hexose phosphates under the influence of adrenaline was observed This was interpreted as an activation of the phosphofructokinase reaction. This effect was not evident in the experiments on colonic muscle

The consumption of high energy phosphate compounds was greater than was evident from the sum of the ATP and CrP reductions of the muscle On simultaneous determination of the changes in the ADP and AMP concentrations it was found that the former nucleotide increased by 0.11 ± 0.025 μmole/g 60 sec after the addition of isoprenaline (Fig 3) The increase in the AMP concentration can probably be ascribed to the reaction 2 ADP $\xrightarrow{\text{myokinase}}$ ATP+AMP On the formation of AMP from ATP, two high energy phosphate bonds are released The total consumption of high energy phosphate compounds can be calculated from the changes of the ATP, ADP, AMP and CrP concentrations to be 0.37 ± 0.04 μeq/g The total concentration of preformed high energy phosphate compounds corresponded to 1.24 μeq/g and therefore approximately 30 % of the preformed content had been consumed 60 sec after the addition of isoprenaline

Dose-response curve after isoprenaline treatment

In order to investigate more closely the relationship between the relaxation and metabolic effects after treatment with isoprenaline the dose response curve for these effects was studied It is evident from Fig 4 that isoprenaline in a concentration as low as 7×10^{-9} g/ml, which after 60 sec had lowered the tension by 18 % induced a significant increase of the phosphorylase *a* activity an increase in the concentration

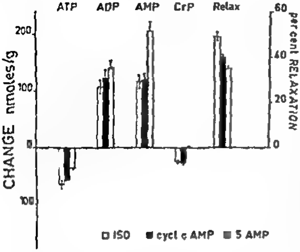

Fig 3 Effect of Isoprenaline (5×10^{-7} g/ml), cyclic AMP (1.1×10^{-6} mole/ml), 5 AMP (1.1×10^{-6} mole/ml) on relaxation and high energy phosphate compounds in colonic muscle 60 sec after the addition of these substances Relaxation per cent = decrease in tension (p) in per cent of initial tension Changes of the metabolite content from the basal value in nmole/g wet weight Mean ± S E M ($n = 6$—12)

of hexose phosphates and a reduction of the ATP and CrP concentrations With an increase of the isoprenaline concentration parallel increases were seen in the relaxing phosphorylase *a* activating and ATP/CrP reducing effects

Effect of adrenergic β receptor blockade

When the relaxing effect of isoprenaline was blocked with sotalol 1.2×10^{-5} g/ml the metabolic effects normally appearing after 60 sec were almost completely inhibited (Table II) For a few metabolites inverse effects were observed Sotalol and isoprenaline in combination thus reduced the G 6-P concentration and tended to increase the concentration of ATP Sotalol in itself had some relaxing effect on the muscle and decreased significantly the ATP concentration

Effect of isoprenaline on carbohydrate-poor muscle

In an earlier study we found that the relaxing effect provoked by β receptor stimulation was inhibited in taenia coli whose carbohydrate reserves had been reduced under anaerobic conditions while the relaxing effect of α receptor stimulations persisted (Andersson and Mohme Lundholm 1969 a) It seemed of interest to study how the metabolic effects induced by β receptor stimulation were influenced under these conditions

The increase in tension after the addition of carbamylcholine was less in the carbohydrate poor specimens than in the normal ones The concentrations of hexose phosphates and ATP in the carbohydrate poor specimens were considerably lower than in normal specimens while the CrP concentrations was the same (Table III) The relaxing effect of isoprenaline (1.6×10^{-7} g/ml) was completely eliminated in carbohydrate poor specimens and isoprenaline induced none of the metabolic effects which occurred in normal muscle (Table III) On stimulation of α receptors with phenylephrine a relaxing effect was still produced Cyclic AMP produced no relaxing effect

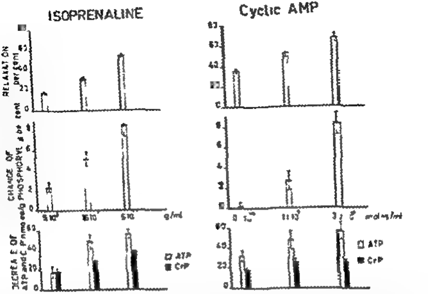

Fig 4 Dose-response curves for the effect of l-isoprenaline (free base) and cyclic AMP on relaxation phosphorylase *a* activity and the content of high energy phosphate compounds in colonic muscle The determinations were performed 60 sec after the addition of these substances Mean±S E.M Isoprenaline tests n=7 Cyclic AMP tests n=5

Effect of isoprenaline in K rich solution

One principal difference between the relaxation induced via α and β receptors was that the former was completely blocked in K rich solution while the latter was only reduced in magnitude In K-rich solution (145 meq/l K) the phosphorylase *a* activity in the muscle was significantly increased in relation to that in normal buffer solution (Table III) Isoprenaline in a concentration of 5×10^{-7} g/ml induced a relaxing effect which was significantly weaker than in normal buffer solution Isoprenaline provoked a phosphorylase *a* activating effect and decreased the concentration of high energy phosphate compounds in K rich solution, but the latter effects were weaker than in normal buffer solution (Table III) The basal concentrations of ATP and CrP were lower however in K treated than in normal muscle specimens

Relaxing and metabolic effects of cyclic AMP

According to Sutherland Butcher and Robinson (1968) some of the metabolic effects of the catecholamines such as the phoshporylase-activating and lipolytic effects are mediated by an increased formation of cyclic AMP In brown adipose tissue noradrenaline induced a reduction of the ATP and CrP contents which could be reproduced by treatment with a combination of cyclic AMP and theophylline (Bewiz Lundholm and Mohme Lundholm 1968) In smooth muscle which is relaxed by catecholamines the formation of cyclic AMP has been found to be increased (Butcher *et al* 1965) Dobbs and Robinson 1968) and cyclic AMP in itself has been shown to relax smooth muscle (Eggena Walter and Schwartz 1968) I

previous study it was found that theophylline which inhibits the enzymatic break down of cyclic AMP, potentiated the relaxing effect induced via β receptor stimulation but not that mediated by activation of α receptors (Andersson and Mohme Lundholm 1969 a) In this study β receptor stimulation increased the cyclic AMP content In view of these findings it was of interest to study to what extent the relaxing and metabolic effects of isoprenaline could be reproduced by cyclic AMP

Cyclic AMP in a concentration of 0 36—3 2×10⁻⁶ mole/ml induced a relaxing effect in colonic muscle and the degree of relaxation was proportional to the dose (Fig 4) After 60 sec all doses had caused a significant decrease of the ATP and CrP concentrations and the two highest doses had also produced significant phosphorylase a activation Cyclic AMP also elevated significantly the concentrations of G 1 P and F 1 6 P

A comparison between the relaxing and metabolic effects of cyclic AMP and isoprenaline showed that cyclic AMP in a concentration of $3\,3\times10^{-6}$ moles/ml induced effects of almost equal magnitude to those exerted by isoprenaline in a concentration of 5×10 g/ml At lower concentrations of cyclic AMP and isoprenaline however some difference in their action was noted Thus isoprenaline in a concentration of $1\,6\times10$ g/ml induced a relaxing effect and reduced the concentrations of ATP and CrP and also activated phosphorylase a Cyclic AMP in a concentration of $0\,36\times10^{-6}$ mole/ml induced no phosphorylase a activation but otherwise effects of equal magnitude to those of isoprenaline

Dibutyryl cyclic AMP has been shown in liver among other tissues to have a stronger phosphorylase activating effect than cyclic AMP and also has a stronger lipolytic and calorigenic effect in adipose tissue (Sutherland *et al* 1968 Beviz *et al* 1968) In experiments on rabbit intestine dibutyryl cyclic AMP in a concentration of 1 08 μmole/ml induced no relaxing effect or stimulation of the phosphorylase activity or other metabolic processes (Table III) Neither did it have any effects in a two times higher concentration Levine (1968) also reported that dibutyryl cyclic AMP had no relaxing effect in rabbit intestine Rabbit colon thus seems to show deviating conditions in relation to other tissues with regard to its sensitivity to cyclic AMP and dibutyryl cyclic AMP We also studied if some other cyclic nucleotides added in the same concentration as cyclic AMP had any relaxing action In a concentration up to 1 1 μmole/ml cyclic guanosine cytidine uridine desoxythymidine or inosine monophosphate had no relaxing action on the rabbit colon

Relaxing and metabolic effects of 5 AMP

Cyclic AMP is hydrolysed in the tissues to 5'AMP by a specific phosphodiesterase (Butcher and Sutherland 1962 It seemed conceivable that after hydrolysis to 5'AMP, cyclic AMP would induce a reduction of ATP by stimulation of the reaction AMP+ATP myokinase 2 ADP Both cyclic AMP and isoprenaline also significantly increased the concentration of 5'AMP in the muscle Fig 3 It was of interest therefore, to make a comparative study of the relaxing and metabolic effects of 5'AMP and cyclic AMP

It is evident from Fig 1 that 5'AMP in a concentration of 1 08 μmole/ml induced a relatively short lasting relaxation which contrasted to the more protracted relaxing effect of cyclic AMP in the same concentration In similar concentrations 5'AMP caused a significantly smaller reduction of the ATP concentration than cyclic AMP In contrast to cyclic AMP, 5'AMP did not stimulate the phosphorylase *a* activity or increase the hexose phosphate concentration and neither did it reduce the concentration of CrP (Table III) On the other hand the ADP concentration in the muscle was increased highly significantly by 5'AMP Thus the increase in ADP after treatment with 5'AMP was significantly higher than after isoprenaline ($\Delta = 0\ 052 \pm 0\ 015$ μmole/g n=6 $P<0\ 02$) despite the fact that the ATP-reducing effect of isoprenaline was significantly stronger than that of 5'AMP ($\Delta = 0\ 052 \pm 0\ 017$ μmole/g $P<0\ 05$) (Fig 3) *These results indicate that the relaxing and metabolic* effects of cyclic AMP (and isoprenaline) cannot be ascribed only to an increased content of 5'AMP

Effect of dinitrophenol on tension and metabolites in colonic muscle from the rabbit

It was considered of interest to study whether a reduction of the ATP concentration of the magnitude produced by isoprenaline and cyclic AMP but induced in a different manner, was associated with a relaxing effect Dinitrophenol (DNP) activates an mitochondrial ATP ase and „uncouples" oxidative phosphorylation (Kelley 1961) and reduces thereby the ATP content of the tissues DNP was used in a concentration of $6\ 6 \times 10^{-8}$ moles/ml After 5 min the muscle specimen whose tension had been increased by carbamylcholine was relaxed by DNP to approximately the same extent as after isoprenaline in a concentration of 5×10^{-7} g/ml The relaxing effect of DNP appeared considerably more slowly however than that of isoprenaline (Fig 1) After 5 min a significant increase of the phosphorylase *a* activity and a significant decrease of the ATP and CrP concentrations had occurred (Table II) In view of the possibility that these effects of DNP might have been induced by the liberation of noradrenaline from the sympathetic nerve endings we studied if they were inhibited by adrenergic β receptor blockade After treatment with sotalol in a concentration of $1\ 2 \times 10^{-6}$ g/ml however the relaxing and metabolic effects of DNP remained unchanged (Table II)

Correlation between relaxation phosphorylase a activation and ATP reduction

Table IV gives the correlations between relaxation and (a) phosphorylase *a* activation and (b) ATP reduction after treatment with different drugs A significant correlation was found between the relaxing and ATP reducing effects of isoprenaline and also between the same effects after cyclic AMP The slopes of the regression lines (b values) were similar In the experiments with 5'AMP the corresponding correlation was also statistically significant and the regression lines showed a similar slope to those of isoprenaline and cyclic AMP

In the experiments with isoprenaline and also in those with cyclic AMP a significant correlation was obtained between relaxation and phosphorylase *a* activation

TABLE IV. Correlation between relaxation in per cent (=100 × decrease of tension in p/initial tension) and decrease of ATP content (μmoles/g wet weight) or change of phosphorylase *a* activity from basal values, b=coefficient of regression, r=coefficient of correlation, n=number of tests

Correlation between relaxation and	n	b	r	p
Isoproterenol 5 × 10^{-7} g/ml 60 sec				
ATP	37	−0.22	−0.60	<0.001
Phosphorylase *a*	21	0.73	0.67	<0.001
Between ATP and phosphorylase *a*	21	−0.17	−0.11	<0.7
Cyclic AMP, 1.1 × 10^{-6} mole/ml 60 sec				
ATP	14	−0.22	−0.65	<0.02
Phosphorylase *a*	10	0.33	0.74	<0.05
5'AMP, 1.1 × 10^{-4} g/ml 60 sec				
ATP	12	−0.33	−0.70	<0.02
Phosphorylase *a*	6	−0.02	−0.11	<0.9
DNP, 6.6 × 10^{-8} mole/ml 5 min				
ATP	12	−0.23	−0.40	<0.2
Phosphorylase *a*	12	0.002	0.07	<0.9

No such significant correlation was found, on the other hand, for 5'AMP and DNP.

The relaxing effect of isoprenaline was correlated both to the ATP reduction and to phosphorylase *a* activation. The phosphorylase *a* activation is an ATP-consuming reaction. It seemed conceivable, therefore, that phosphorylase *a* activation and ATP reduction might have been correlated and could have explained the double correlation of the relaxation to ATP reduction and phosphorylase *a* activation. This possibility was refuted, however, by the fact that in the isoprenaline experiment the ATP reduction and phosphorylase *a* activation were not significantly correlated (Table IV). Further evidence against this assumption was the fact that cyclic AMP in a low concentration reduced the ATP concentration without activating phosphorylase *a* (Fig. 2).

Discussion

Relaxing effect induced via stimulation of adrenergic α receptors

The relaxing effect which was induced in colonic muscle from rabbit via stimulation of adrenergic α receptors appeared after a short latency period. Initially it was accompanied by a decrease of the lactate content of the muscle. After 3 min a significant decrease of the phosphorylase *a* activity, a decrease of the concentrations of hexose phosphates and lactate in the muscle and an increase of the concentration of high-energy phosphate compounds were also evident. There was a decrease of the cyclic AMP content.

These metabolic effects were probably dependent upon a stimulation of adrenergic α receptors, since they were inhibited by dibenamine. As they did not become manifest until the relaxation had reached a maximum, we consider it improbable that the metabolic effects could have induced the relaxation.

Bueding *et al* (1967) found in experiments on taenia coli from the guinea pig that the relaxing effect of adrenaline was accompanied by an increase in the ATP concentration in the muscle but that there was no stimulation of the phosphorylase *a* activity or lactate production The effect was also observed in carbohydrate poor muscle In similar experiments on guinea pig taenia coli Bueding *et al* (1966) found that the relaxing effect of adrenaline was not associated with any increase of the concentration of hexose phosphates in the muscle and that there was a relatively negligible increase in the content of cyclic AMP

In previous experiments we found that the relaxing effect of adrenaline in taenia coli from the guinea pig was induced principally via a stimulation of adrenergic α receptors Only in a high concentration did adrenaline also stimulate β receptors (Andersson and Mohme Lundholm 1969 a) It is therefore probable that the ATP increase observed by Bueding *et al* (1967) after adrenaline treatment of taenia coli from guinea pig in the absence of a stimulatory effect on the carbohydrate metabolism, was also an α receptor effect and had the same mechanism which we observed in rabbit colon on stimulation with phenylephrine

Bulbring and Golenhofen (1967) found in taenia coli from the guinea pig that adrenaline decreased the oxygen consumption of the muscle and had no influence on its lactate production In our experiments phenylephrine reduced both the lactate content and the content of hexose phosphates in colonic muscle The increase of the ATP content observed on stimulation of adrenergic α receptors are therefore not associated with metabolic reactions leading to an increased synthesis of ATP It is more probable that these effects were due to a reduced utilization of ATP following the relaxation of the muscle and a secondary inhibition of ATP synthesizing reactions

Relaxing effect induced via adrenergic β receptors

The relations between metabolic processes and relaxation induced via catecholamines were completely different when the relaxations was induced via adrenergic β receptors than when it was mediated by adrenergic α receptors Since in previous studies on the metabolic effects of the catecholamines in smooth muscle no identification has been made of the receptors type which has been stimulated it is not surprising that different investigators have arrived at diametrically opposite results depending upon which muscle specimen has been studied

Mechanism for β receptor induced relaxation

Our results indicate that there was an intimate relationship between the relaxing effect and metabolic actions of isoprenaline in smooth muscle The nature of this relationship is an interesting question Is there a causative connection between relaxation and metabolic effects or are these effects parallel phenomena?

If relaxation induced via adrenergic β receptors were only a parallel phenomenon with the meabolic processes it should be possible to find conditions in which these phenomenon could be dissociated This has not been possible hitherto however The relaxing effect of isoprenaline showed the same time response and dose response

curves (Fig 2) as the metabolic effects Both types of effects were blocked by an adrenergic β receptor blocking agent and were weakened in solution with a high K^+ content In carbohydrate poor muscle both the relaxing and metabolic effects were blocked Significant correlations was found both between the degree of relaxation and ATP reduction and phosphorylase *a* activation Drugs which activated phosphorylase *a* and decreased the ATP concentration to the same extent as isoprenaline such as cyclic AMP or DNP, also induced a relaxing effect of equal magnitude Dibutyryl cyclic AMP, which in rabbit colon had no metabolic effects induced no relaxation either

In those cases where a dissociation between relaxation and stimulation of phosphorylase *a* activation and the carbohydrate metabolism was demonstrated (Bueding *et al* 1962 Bueding *et al* 1966) it seems probable from the results of this and a previous study (Andersson and Mohme Lundholm 1969 a) that the relaxation was induced wholly or partly by adrenergic *a* receptors Both in the present investigation and in previous studies on smooth muscle in which the relaxation was mainly induced via adrenergic β receptors a covariation was found under different conditions between the relaxing and the lactate producing effect of the catecholamines (Mohme Lundholm 1953 1957 1960 1962) their glycogenolytic effects (Lundholm and Mohme Lundholm 1957) and their phosphorylase *a* activating actions (Brody and Diamond 1967)

Several findings point to the possibility that cyclic AMP may be a mediator of the relaxation and metabolic effect induced by β receptor stimulation. In uterine muscle where the relaxation is mainly induced via adrenergic β receptors (Miller 1967) the catecholamines increased the concentration of cyclic AMP sixfold (Butcher *et al* 1965 Dobbs and Robinson 1968) while in taenia coli from the guinea pig where the relaxation is mainly induced via adrenergic *a* receptors (Andersson and Mohme Lundholm 1969 a) the elevating effect of adrenaline on the cyclic AMP concentration did not exceed 40 % even when high doses of adrenaline were used (Bueding *et al* 1966) In our experiments on rabbit colon β receptor stimulation increased the cyclic AMP content whereas the *a* receptor mediated relaxation was associated with a decrease of the cyclic AMP content Drugs which have been found to block the stimulation by catecholamines of the formation of cyclic AMP such as sotalol (Bevaz and Lundholm 1970) inhibited the β receptor induced relaxation Drugs which have been claimed to inhibit the enzymatic breakdown of cyclic AMP such as theophylline (Butcher and Sutherland 1962) and puromycin (Appleman and Kemp 1966) potentiated the relaxing effect of isoprenaline (Andersson and Mohme Lundholm 1969 a) Cyclic AMP induced the same effects as isoprenaline in colonic muscle from the rabbit namely relaxation, phosphorylase *a* activation and reduction of the concentration of high energy phosphate compounds All these results support the hypothesis that cyclic AMP may be a mediator of the relaxing and metabolic effects of isoprenaline in smooth muscle

In the present study the β receptor induced relaxation was preceded by an in

crease in the phosphorylase a activity and increased concentrations of hexose phosphate and lactate in the muscle. When the relaxation in per cent of the total tension was correlated to the increase in the phosphorylase *a* activity in all experiments with isoprenaline, a significant correlation was found (Table IV). In [illegible] it was demonstrated that ACTH relaxed rabbit colon through adrenergic [illegible]. This effect was associated with an increased cyclic AMP formation, a phosphorylase *a* activation and stimulation of the carbohydrate metabolism. ACTH did not reduce the ATP content of the muscle (Anderson and Mohme-Lundholm 1970). These and many other results (Mohme-Lundholm 1953, 1956, 1960, 1962) indicate a relationship between relaxation and stimulation of the carbohydrate metabolism.

Other results have shown, however, that an increased carbohydrate metabolism alone cannot be the cause of the relaxation. In K^+-rich buffer solution neither lactic acid nor a decrease in pH induced a relaxing effect in smooth muscle (Svedmyr, Mohme-Lundholm and Vamos 1967). Isoprenaline still caused relaxation in K^+-rich solution, however, even though the effect was weakened in comparison with experiments with ordinary buffer solution. The ATP content was decreased by isoprenaline in K^+-rich solution, however (Table III).

A significant correlation was found between the degree of relaxation and reduction of ATP (Fig. 5). In a low concentration cyclic AMP, like 5'AMP, relaxed the colonic muscle and decreased the ATP concentration without any phosphorylase *a* activation. The phosphorylase *a* activating and ATP reducing actions of isoprenaline were not correlated (Table IV). These results indicate that the decrease in the concentration of high energy phosphate compounds induced by isoprenaline was probably also of importance for its relaxing effect. In an attempt to determine whether an ATP reduction of the magnitude induced by isoprenaline and cyclic AMP was accompanied by a relaxing effect, a study was made of the effect of DNP. On a decrease of ATP of approximately the same order of size as that induced by isoprenaline and cyclic AMP, DNP also decreased the tension of the muscle to approximately the same extent as these drugs. One finding which contradicts the possibility that the relaxation can be ascribed solely to a reduction of the ATP content was that 10 min after the addition of isoprenaline, when the relaxation [illegible] remained unchanged, the ATP content had increased significantly (Fig. 4). Also in vascular muscle it was found previously after blockade of adrenergic receptors that adrenaline had a relaxing effect and that the ATP content of the muscle had increased after 7.5 min (Bevz and Mohme-Lundholm 1965). These results indicate that isoprenaline probably via cyclic AMP formation can initiate two separate metabolic reactions leading to relaxation, i.e. phosphorylase *a* activation and an ATP consuming reaction.

Two explanations for the relationship between relaxation and ATP reduction may be discussed. Either the relaxation was due to the fact that less ATP was available for the contraction process or the relaxation was a result of an energy-demanding process initiated by isoprenaline. Bülbring (1960) has suggested that in taenia coli of the guinea pig adrenaline may stimulate an electrogenic Na pump which [illegible]

hyperpolarisation of the cell membrane and relaxation

It is unlikely that this mechanism could be of importance for the β receptor induced relaxation in our experiments Firstly this relaxing effect was not blocked by digitalis glucosides which specifically block the Na^{+}—K^{+} pump in the cell membrane (Andersson and Mohme Lundholm 1969 a), and secondly isoprenaline also relaxed colonic muscle in K^{+}-rich buffer solution where stimulation of the Na^{+} pump probably plays no role in the membrane potential Finally Johansson *et al* (1967) found that β receptor induced relaxation in vena cavae from the rat was accompanied by an increase in the electrical activity of the cell membrane Since however, both adrenergic α- and adrenergic β receptor stimulation gradually leads after a certain latency period to an increase in the ATP content this may result secondarily in stimulation of the Na^{+} pump

In further experiments we found that the ATP-reducing effect of isoprenaline was inhibited in Ca -free solution *i e* that the ATP-ase activating action of isoprenaline was Ca dependent One explanation for this is that isoprenaline stimulated an ATP-utilizing Ca^{++} pump (Andersson and Mohme Lundholm 1969 b) The role of Ca in the relaxing and metabolic effects of the catecholamines will be discussed in a following paper

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References

Aaberg G E Mohme Lundholm and N Vamos The effect of H^{3} and lactate ions on the electrical activity and content of high energy phosphate compounds of taenia coli from the guinea pig *Acta physiol scand* 1967 69 129—133

Adam H Adenosine 5 *Methods of enzymatic analysis* (H

Ahlquist R P and B *J Pharmacol exp Ther* 1959 127 [illegible]

Andersson R and E Mohme Lundholm Metabolic actions associated with stimulation of α and β receptors for adrenaline in smooth muscle *Brit J Pharmacol* 1968 34 201P

Andersson R and E Mohme Lundholm Studies on relaxing actions mediated by stimulation of adrenergic α and β receptors in taenia coli of the rabbit and guinea pig *Acta physiol scand* 1969 a 77 372—384

Andersson R and E Mohme Lundholm Relaxing and metabolic actions of ACTH in rabbit colon *Acta physiol scand* 1970 b (In press)

Andersson R and E Mohme Lundholm Role of Ca in metabolic and mechanical effects of drugs in intestinal smooth muscle *Abstr 4 th Int Congr Pharmac* (Basel 1969 b c p 332

Appleman M M and R B Kemp Puromycin a potent metabolic effect independent of protein synthesis *Biochem biophys Res Commun* 1966 24 564—568

Bergmeyer H V and H Klotch D Glucose 1 phosphate In *Methods of enzymatic analysis* (H V Bergmeyer ed Acad Press 1963 pp 131—133

Beviz A and L Lundholm Influence of adrenaline and an adrenergic β receptor blocking agent (Sotalol) on the cyclic AMP content of rat diaphragm *Life Sci* 1970 (In press)

Beviz A L Lundholm and E Mohme Lundholm Cyclic AMP as a mediator of hormonal metabolic effects in brown adipose tissue *Brit J Pharmacol* 1968 34 198 199P

Beviz, A and E Mohme Lundholm Influence of dihydroergotamine and adrenaline on the concentration of glucose 6 phosphate fructose 6 phosphate adenosine triphosphate and [illegible] artery *Acta physiol scand* 1965 65 289—291

Be [illegible] Svedmyr The effect of adrenaline on the carbo- *Acta physiol scand* 1967 69 213 217

Bu [illegible] and H Kuriyama Lack of activation of phosphorylase by adrenaline during its physiological action on smooth muscle *Nature* (Lond) 1962 19 944—946

and E W SUTHERLAND Effect of
-tose phosphates in intestinal smooth
—178
d H KURIYMA The effect adrenaline
on the adenosinetriphosphate and creatine phosphate content of intestinal smooth muscle *J Physiol* (Lond) 1967 *193* 187—212
BRODY TH M and J DIAMOND Blockade of the biochemical correlates of contraction and
1967 139 772—780
BU
BU
1250
BUCHER TH and J J HOHORST D hydroxyacetone phosphate fructose 1 6 diphosphate and D Glyceraldehyde-3 phosphate In *Methods of enzymatic analysis* (H V Bergmeyer ed) Acad Press. 1963 pp 246—252
zymology (S P Colowich and N O Kaplan eds) Acad Press N Y 1955 *1* 200—205
DOBBS J W and G A ROBINSON Functional biochemistry of β receptors in the uterus *Fed Proc* 1968 *27* 352
EGGENA P R WALTER and I L SCHWARTZ Contractile response in vitro of the urinary bladder of the toad Bufo Marinus to neurohypophyseal hormones theophylline and cyclic 3 5 AMP *Fed Proc* 1968 *27* 444
HOHORTS H J D Glucose-6 phosphate and D Fructose 6 phosphate In *Methods of enzymatic analysis* (H V Bergmeyer ed) Acad Press 1963 pp 134—138
JOHANSSON B O JONSSON J AXELSSON and B WAHLSTROM Electrical and mechanical characteristics of vascular smooth muscle response to noradrenaline and isoproterenol *Circulat Res* 1967 *21* 619—633
KAKIUCHI S and T W RALL A procedure for the estimation of adenosine 3 5 phosphate in tissues 1970 (In press)
LAMPRECHT W and I STEIN Adenosine 5 triphosphate determination with hexokinase and
LUNDHOLM L and E MOHME LUNDHOLM Length at inactivated contractile elements length tension diagram active state and tone of vascular smooth muscle *Acta physiol scand* 1966 *68* 347—359
LUNDHOLM L E MOHME LUNDHOLM and N SVEDMYR Physiological interrelationships Introductory remarks *Pharmacol Rev* 1966 *18* 255—272
LUNDHOLM L E MOHME LUNDHOLM and M VAMOS Lactic acid assay with L(+) lactic acid dehydrogenase from rabbit muscle *Acta physiol scand* 1963 *58* 243—249
MILLER J W Adrenergic receptors in the myometrium *Ann N Y Acad Sci* 1967 *139* 788—798
MOHME LUNDHOLM E The mechanisms of the relaxing effect of adrenaline on smooth muscle *Acta physiol scand* 1953 *29* Suppl 108 1—56
MOHME LUNDHOLM E Mechanisms of the relaxing effect of adrenaline on bovine coronary vessels *Acta physiol scand* 1957 *38* 255—264
MOHME LUNDHOLM E The association between the relaxing and the lactic acid stimulating effects of adrenaline *Acta physiol scand* 1960 *48* 268—275
MOHME LUNDHOLM E Lactic acid production and adrenaline reversal in experiments in isolated smooth muscle *Acta physiol scand* 1962 *55* 225—230
MOHME LUNDHOLM E Smooth phosphorylase and enzymes affecting its activity *Acta physiol scand* 1963 *59* 74—84
SUTHERLAND E W R W BUTCHER and A ROBINSON Some aspects of the biological role of adenosine 3 5 monophosphate (cyclic AMP) *Circulation* 1968 *37* 279—306

Acta physiol. scand 1970. 79. 262—271

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Effects of Varied Cerebrospinal Fluid Pressure on Cerebral Blood Flow in Dogs

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Abstract

HAGGENDAL E, J LOFGREN, N J NILSSON and N N ZWETNOW *Effects of varied cerebrospinal fluid pressure on cerebral blood flow in dogs* Acta physiol scand 1970 *79* 262—271

Effects of short term variations in cerebrospinal fluid pressure on cerebral blood flow in dogs were measured with the radioactive krypton clearance technique of Lassen *et al* The cerebrospinal fluid pressure was increased stepwise by infusion of artificial cerobrospinal fluid the pressure range from −15 to +150 mm Hg being investigated The cerebral blood flow remained virtually stable when the induced cerebrospinal pressure was lower than about 100 mm [illegible] tive roles of these two regulating mechanisms is given

By being enclosed in a rigid fluid containing box, *i e* the cranial cavity the cerebral vessels are directly exposed to a hydrostatic pressure which can vary to a considerable degree During an increase of the cerebrospinal fluid pressure, at least the venous part of the intracranial vascular bed will be compressed and the cerebral perfusion pressure accordingly diminished In the absence of compensatory factors any increase of the cerebrospinal fluid pressure would lead to a corresponding decrease of the cerebral blood flow There are a few experimental studies which indicate a tendency for the cerebral blood flow to remain constant in spite of a moderate increase in cerebrospinal fluid pressure (Noell and Schneider 1948 Green Rapela and Conrad 1963 Shulman and Verdier 1967 see also Lassen 1959) this phenomenon is known to take place when the perfusion pressure is reduced by a lowering of the arterial pressure The effectiveness of different compensatory mechanisms as well as the relationship between them seem however still to be unknown

The present study was undertaken in order to determine the quantitative relationship between the cerebral blood flow (CBF and the cerebrospinal fluid pressure in

the range from negative values (−15 mm Hg) up to the arterial pressure level Special attention was given to the problem of the relative importance of a reduction of the vascular resistance and an increase of the systemic blood pressure, respectively, for the maintenance of flow when the perfusion pressure is reduced The effect of variation of the arterial carbon dioxide tension on the cerebral blood flow during cerebrospinal hypertension was also studied Preliminary reports of these findings have been given in 1966 (Haggendal *et al* 1967, 1969)

Material and methods

The study was performed on mongrel dogs with body weights between 6 and 16 kg, anesthetized ... doses of 3—4 mg ... (Celocurin klorid, ... be by means of an ... s ordinarily used in order to prevent atelectasis The rectal temperature was kept constant (37—38° C) by means of an electric heating pad Clotting was prevented by intravenous administration of heparin ...

... determination of the arterial pH and carbon dioxide tension according to the micro method of ... ant fluctua ... an experi

Blood from the superior sagittal sinus, representing predominantly the cerebral cortex (Hirsch *et al* 1961, Haggendal Nilsson and Norback 1965 Haggendal and Norback 1966), was used ...

... above or below the insertion point of the needle During the passage from the fluid container to the cisterna magna the fluid was heated to body temperature by passage through a water bath regulated by a thermostat Through the other branch of the needle the cerebrospinal fl... orded by means of an induc ... horizontal plane through the ... n as the reference level An ... ressure in the femoral artery

In control experiments simultaneous pressure registrations were carried out in the sub- ...

... ference between the arterial and the cerebrospinal mean pressures

By elevating the fluid container to different levels above the reference point the pressure of the cerebrospinal fluid of the animal could be changed to any desired value in 10—15 sec and kept constant for hours

... ordance with the inert gas elimination method ... also Haggendal *et al* 1965 Hoedt Rasmussen, ... injection of radioactive krypton A thin poly... hrough a small side branch of the left subclavian artery and advanced under visual control from the subclavian 5—8 cm up into the vertebral artery which thus did not have to be ligated around the catheter The catheter was filled with heparinized saline solution and sluiced at intervals The isotope Kr^{85} (Radio-

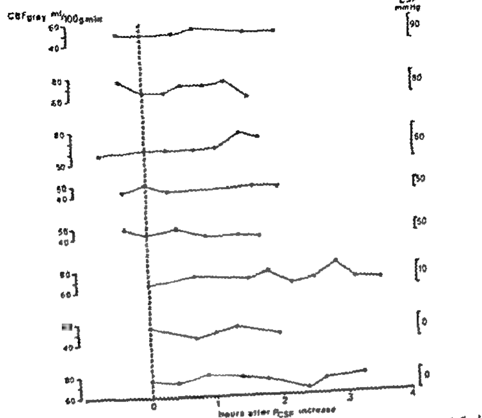


FIG 1 Long term stability of blood flow in cerebral grey matter when cerebrospinal fluid pressure is kept constant at different levels

chemical Centre Amersham England) was dissolved in saline The volume of Kr[85] solution injected for each determination of CBF was 0.5—2 ml and the injections lasted about 1—3 sec The activity of the gas was measured with a scintillation detector (2" NaI crystal), coupled to a ratemeter Nukleonikinstruments AB Goteborg) and recorded on a Beckman potentiometer writer the time constant of both being 1 sec The detector was placed over the skull so that the greater part of the brain was seen by the crystal The influence of extracerebral activity was reduced as much as possible by lead collimation To avoid a rise of background activity in the operation room the expired air of the animal was eliminated through a tube attached to the outlet of the respiratory pump

The elimination curves which were recorded for at least 15 min were plotted semilogarithmically and dissolved in the ordinary way into two exponential functions the faster of which is regarded as an expression of the blood flow in the cerebral grey matter In the following only these cortical flow values are discussed

Results

Cortical blood flow during prolonged periods of increased cerebrospinal fluid pressure

In one series of animals the reproducibility of the cortical flow determinations under the conditions of this study was investigated by repeated flow measurements during periods of 1 1/2—3 1/2 hrs with the cerebrospinal fluid pressure kept constant at various levels As demonstrated in Fig 1 no systematic flow change occurred during

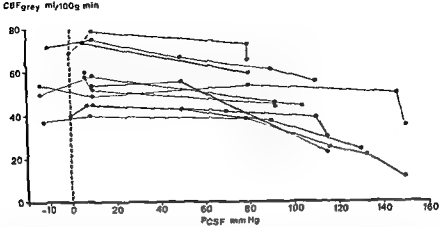


Fig 2 Cortical blood flow at different induced cerebrospinal fluid pressure levels in the range −15 mm to +150 mm Hg, Pa_{CO_2} 30—40 mm Hg The cortical blood flow is maintained fairly constant at negative CSF pressures and at positive pressures below 80 mm Hg, showing a progressive decrease at higher pressure levels

this time, and the random variation amounted to about 10 % or in isolated instances 20 % of the flow value

Cortical blood flow during stepwise increase in cerebrospinal fluid pressure (Pa_{CO_2} = 30—40 mm Hg)

Fig 2 demonstrates the values for cortical blood flow when cerebrospinal fluid pressure was raised, in some cases after a preceding period of negative pressure In the range −15 mm Hg to 80—100 mm Hg the cortical blood flow was virtually unchanged At higher pressure levels a pronounced progressive flow reduction occurred Negative CSF pressure did not seem to affect the cortical blood flow

The effect of the cerebrospinal fluid pressure on cerebral hemodynamics is more evident when the blood flow is presented as a function of the cerebral perfusion pressure, i e the mean arterial blood pressure minus the cerebrospinal fluid pressure, which is considered equal to the intracranial venous pressure Fig 3 shows that the blood flow remained stable or showed a very moderate reduction until the perfusion pressure was reduced below 30—50 mm Hg — a manifestation of a well functioning autoregulation of the blood flow When the perfusion pressure was lowered further (due to an induced cerebrospinal fluid pressure of usually over 100 mm Hg) there was a pronounced decrease of the cortical blood flow The lowest flow values obtained during intracranial hypertension were 10—20 ml per min and 100 g brain tissue When the cerebrospinal fluid pressure was raised still further during these conditions, usually a rapid fall of the systemic blood pressure occurred and the animals succumbed

When the cerebral blood flow decreased during the influence of the raise

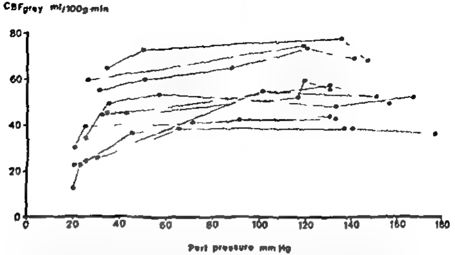
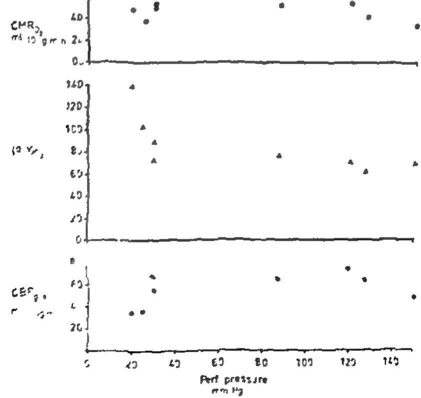


Fig 3 Cortical blood flow during stepwise reductions of the cerebral perfusion pressure (same material as in Fig 2) The blood flow is maintained *practically unchanged until the perfusion* pressure is reduced below about 40 mm Hg, whereupon it rapidly decreases



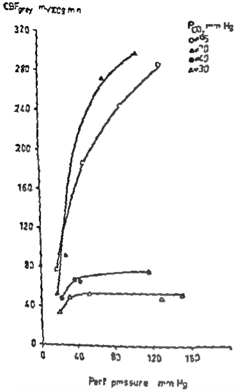


Fig 5

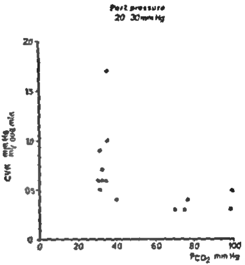


Fig 6

Fig 5 Influence of reduced cerebral perfusion pressure on cortical blood flow at different arterial P_{CO_2}. In principle the curves have the same shape as the composite curve shown in Fig 3. Higher flow values and diminishing autoregulatory tendency are seen concomitantly with rising P_{CO_2}.

Fig 6 ... resistance at perfusion pressure values of ... dues at higher arterial P_{CO_2} levels suggest ... range where maximal dilatation of the

cranial pressure there was an increase of the arterio-venous oxygen difference tending to maintain the cerebral oxygen consumption constant. An example of this is seen in Fig 4

Effects of raised intracranial pressure on CBF in hypercapnic animals

The well known vasodilating effect of hypercapnia and the vasoconstricting effect of hypocapnia are also evident during high intracranial pressure. These effects are demonstrated in Fig 5. Also at low perfusion pressures when flow autoregulation was not evident, the flow was higher at high than at low carbon dioxide tensions. This vasoactive effect of carbon dioxide is presented in Fig 6 at a perfusion pressure as low as 20—30 mm Hg when the autoregulatory ability can be assumed to be abolished

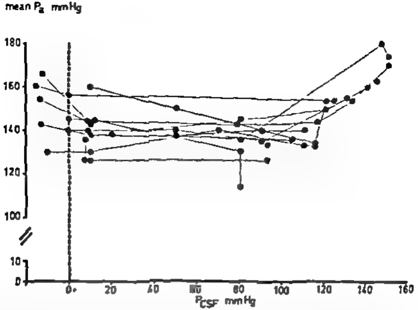


Fig 7 Relationship between mean arterial pressure and cerebrospinal pressure Mean arterial pressure remains constant during progressively rising cerebrospinal pressure until P_{CSF} approaches values about 100 mm Hg At higher intracranial pressure levels the mean blood pressure shows compensatory increases up to over 170 mm Hg (Cushing reaction) Moderate blood pressure increases were also seen at induced negative P_{CSF} levels

Compensatory blood pressure rise at increased intracranial pressure

The other mechanism contributing to the maintenance of the blood flow besides the autoregulatory vasodilatation is the rise in systemic arterial pressure (Cushing effect), tending to prevent the cerebral perfusion pressure from decreasing This tendency is illustrated in Fig 7 where the effect of the cerebrospinal fluid pressure on the mean arterial blood pressure is presented At intracranial pressures from 0 to about 100 mm Hg the arterial blood pressure remains almost constant at a value around 140 mm Hg When the cerebrospinal fluid pressure is increased further, however the systemic blood pressure tends to rise sometimes to more than 170 mm Hg Also at negative intracranial pressures which did not influence the cerebral blood flow (Fig 2) a rise of the systemic arterial blood pressure was usually observed

Discussion

The intracranial pressure range investigated from -15 to $+150$ mm Hg comprises most of the cerebrospinal fluid pressures which are of clinical interest In the normo- or hyperventilated animals there was no obvious cerebral blood flow reduction until the intracranial pressure exceeded 80—100 mm Hg (Fig 2) These pressure values are remarkably high and of the same order of magnitude as the highest pressure values generally observed in patients (Lundberg 1960) As there was no systematic

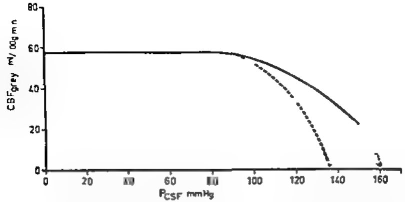


Fig 8 A schematic CBF/P_{CSF} curve illustrating the relative importance of peripheral vasodilatation and compensatory systemic arterial pressure rise in the maintenance of the cerebral blood flow during progressive intracranial hypertension The broken line represents deduced blood flow values based on the actual peripheral resistance in the absence of the Cushing effect

change in the arterial pressure when the cerebrospinal fluid pressure was less than 100 mm Hg the flow constancy had to be effected entirely by a cerebral vasodilatation The vascular response to the high intracranial pressure is presumably of the same kind as the cerebral blood flow autoregulation which occurs at arterial blood pressure reduction (Hirsch and Korner 1964 Harper 1965 Häggendal 1965) Thus in both cases this mechanism tends to preserve the cerebral blood flow when the cerebral perfusion pressure is reduced

The other compensatory mechanism which tends to reduce the effect of the intracranial pressure rise on the cerebral perfusion pressure is an increase in the systemic blood pressure This rise in arterial blood pressure often called the Cushing effect did not occur until the intracranial pressure was over about 100 mm Hg This observation is in agreement with earlier findings (Naunyn and Schreiber 1881 Cushing 1901 Noell and Schneider 1948 Sagawa *et al* 1961 Dickinson and McCubbin 1963 Richardson Fermoso and Pugh 1965 Sagawa 1967) The increase in arterial blood pressure however, did not maintain the cerebral blood flow at the control level the flow had already begun to decrease before the rise in blood pressure occurred It seems therefore reasonable to assume a causal relationship between the flow reduction and the rise in the systemic blood pressure However the cerebral metabolic rate of oxygen measured in 3 animals remained practically constant even when the perfusion pressure was lowered as much as to 20 mm Hg The methods used in our studies cannot however exclude the possibility of minor focal cerebral ischemia

The magnitude of the blood pressure rise in our experiments was rather modest In most cases the pressure increase was 20—30 mm Hg resulting in an arterial mean pressure of maximally 170 mm Hg But even the slight increase in cerebral perfusion pressure which was established by the arterial pressure increased has a definite

importance in diminishing the degree of the cerebral ischemia This effect is schematically illustrated in Fig 8 The upper line in this figure connects the means of the flow values given in Fig 2 and the dotted line depicts in rough terms the flow values which would have been obtained if the arterial blood pressure increase, as illustrated in Fig 7, had not occurred Thus, the flow values in the lower curve are calculated from the perfusion pressure, which would have been present at a constant arterial pressure of 140 mm Hg and from the actually observed resistance values

As was mentioned earlier the animals usually succumbed when induction of high cerebrospinal fluid pressure reduced the cerebral blood flow below 10—20 ml/100 g min It is evident that such a low flow is insufficient for the metabolic needs of the cerebral tissue In this connection the hypothesis of a 'critical closing pressure may be considered since the pressure flow relation curves seem to have a tendency to intersect the pressure axis at a value of just below 20 mm Hg irrespective of the carbon dioxide pressure

The influence of high arterial carbon dioxide tension on the cerebral vessels when the perfusion pressure was reduced to between 20 and 30 mm Hg (Fig 6) suggests that in spite of the extreme perfusion pressure reduction, with presumably exhausted autoregulatory vasodilatation, the cerebral vessels are still capable of further dilatation. These results, which are at variance with some earlier findings (Harper 1965), have been substantiated by more recent investigations (Ekstrom Jodal *et al* 1969) They seem to indicate that the mechanisms involved in cerebrovascular dilatation during the influence of increased carbon dioxide tension and during the influence of increased cerebrospinal fluid pressure are not entirely identical

References

Bjure J and N J Nilsson Spectrophotometric determination of oxygen saturation of hemoglobin in the presence of carboxyhemoglobin *Scand J clin Lab Invest* 1965 *17* 491—500

Cushing H Concerning the definite regulatory mechanism of the vaso-motor centre which controls blood pressure during cerebral compression *Bull Johns Hopk Hosp* 1901 *12* 290—292

Dickinson C J and J W McCubbin Pressor effect of increased cerebrospinal fluid pressure and vertebral artery occlusion with and without anesthesia *Circulat Res* 1963 *12* 190—202

Drabkin D L and J H Austin Spectrophotometric studies II Preparations from washed blood cells nitric oxide hemoglobin and sulfhemoglobin *J biol Chem* 1935 *112* 51—65

Ekstrom Jodal B E Häggendal N J Nilsson and B Norback Changes of the transmural pressure — the probable stimulus to cerebral blod flow autoregulation *Proc Int Symp on Cerebral Blood Flow Main 1969* To be published by Springer Berlin

Et [illegible] Studies of [illegible] *J Neurosurg* [illegible]

Green H D [illegible] Rapela and M C Conrad Resistance (conductance) and capacitance phenomena in terminal vascular beds In *Handbook of Physiology* ed W F Hamilton and P Dow Amer Physiol Soc Washington 1963 *Circulation* II 935 960

Häggendal, E Blood flow autoregulation of the cerebral grey matter with comments on its mechanism. *Acta neurol scand* 1965 Suppl *14* 104—110

Häggendal, E J Lofgren N J Nilsson and N Zwetnow Die Gehirndurchblutung bei experimentellen Liquordruckänderungen *Acta neurochir* (Wien) 1967 *16* 163

HÄGGENDAL, E, J LÖFGREN, N J NILSSON and N ZWETNOW, Influence of induced changes in the cerebrospinal fluid pressure on the cerebral blood flow of dogs *Research on the*

HIRSCH, H, U GLEICHMANN, H KRISTEN and V MAGAZINOVIC, Über die Beziehung zwischen O_2-Aufnahme des Gehirns und O_2-Druck im Sinusblut des Gehirns bei uneingeschränkter und ... 213—222

... ion der Gehirngefässe.

... cerebral blood flow in ... *Circulat Res* 1966 *18* 237—247

L... 183—238

LASSEN, N A, K HOEDT-RASMUSSEN, S C SORENSEN, E SKINHOJ, S CRONQUIST, B BODFORSS and D H INGVAR, Regional cerebral blood flow in man determined by krypton[85] *Neurology* (Minneap) 1963 *13* 719—727.

LUNDBERG N, Continuous recording and control of ventricular fluid pressure in neurosurgical practice *Acta psychiat scand* 1960 *36* Suppl *149* pp 193

NAUNYN, B and J SCHREIBER, Über Gehirndruck *Naunyn-Schmiedebergs Arch exp Path Pharmak* 1881 *14* 1—112

NOELL W and ... liquordruck-

... intracranial ... —322

... In *Physical* ... Reeve and A. C Guyton W B Saunders Co

SA... C GUYTON, Dynamic performance and stability of cereb... 1961 *201* 1164—1172

... stance changes in response to cerebro- ... —1088

... P ASTRUP A micro method for deter- ... and standard bicarbonate in capillary

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Prolonged Cerebral Hyperemia after Periods of Increased Cerebrospinal Fluid Pressure in Dogs

By

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Abstract

HAGGENDAL, E, J LOFGREN, N J NILSSON and N N ZWETNOW *Prolonged cerebral hyperemia after periods of increased cerebrospinal fluid pressure in dogs* Acta physiol scand 1970 *79* 272—279

In [illegible] dogs the cerebral blood flow was measured with the intra arterial radioactive injection technique during and after the application of a high cerebrospinal fluid pressure by infusion of artificial cerebrospinal fluid into the subarachnoid space Increased cerebral blood flow was observed in most cases when the cerebral perfusion pressure was restored after a period with cerebrospinal fluid pressure over 50 mm Hg The hyperemia always occurred if during the period of high intracranial pressure the blood flow had been reduced below the control value In a few cases it appeared also without a preceding flow reduction In almost all cases there was a marked tendency to normalization of the blood flow after the initial period of hyperemia No chemical factors in the artificial cerebrospinal fluid could be made responsible for the phenomenon described which [illegible] attributed to a disturbance of cerebrovascular function impairing the ordinary flow autoregulation

Temporary arrest or reduction of the circulation through an organ [illegible] generally followed by [illegible] period of increased blood flow — reactive hyperemia This hyperemia is easily provoked in the myocardium skeletal muscle and skin, in the gut or the kidney it is seen to a much smaller extent (Barcroft 1963, Gregg *et al* 1963 Greenfield 1963) A satisfactory explanation of the mechanisms and causes of the hyperemia is still lacking

As reported earlier (Haggendal *et al* 1967 1969) we have observed a large and long lasting cerebral hyperemia after periods of increased cerebrospinal pressure in dogs The elicited hyperemia reaction could last for more than one hour, and its peak value sometimes amounted to several times the control flow in periods of normal cerebrospinal fluid pressure

The present paper gives a more complete description of our findings concerning the cerebral hyperemia after varying periods of increased cerebrospinal fluid pressure

Material and methods

... between 6 and 16
... of 40—50
... Mechanical
... The body
temperature as measured in the rectum was maintained at 37—38° C with the aid of an electric heating pad After completion of the operative preparation the animals were immobilized with Celocurin klorid (Vitrum), which was given during the experiment in supplementary doses Clotting was prevented by intravenous administration of heparin. A femoral artery was cannulated for blood sampling and the recording of arterial pressure Cerebral ...

matter are presented ...

to different cerebrospinal fluid pressures (Pcsf) for varying periods of time (stress duration) ...

fluid pressure with subsequent pressure reduction In four experiments the procedure was repeated two or more times

Results

Cerebrospinal fluid pressures were induced to values between 50 and 140 mm Hg The corresponding perfusion pressures i.e the differences between the mean arterial pressure and the cerebrospinal fluid pressure varied between 130 and 0 mm Hg At cerebrospinal pressure levels of 100 mm Hg and more, a reactive increase in blood pressure—the Cushing effect—was usually observed, and amounted to 25—45 mm Hg The duration of the cerebrospinal pressure increase (stress duration) was generally between 15 and 60 min In two dogs stress durations of 1/4 to 5 min were applied

Fig 1 illustrates a typical experiment The upper curves show the cerebrospinal fluid pressure, the mean arterial blood pressure and the resulting perfusion pressure of the brain In this experiment the cerebrospinal fluid pressure was gradually in-

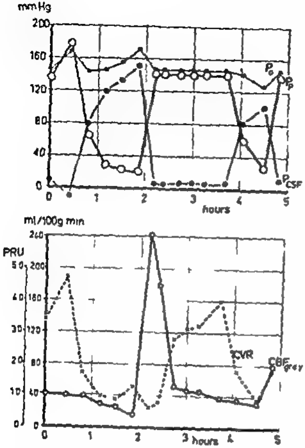

Fig 1

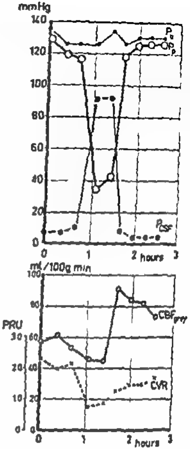

Fig 2

Fig 1 *Upper part* mean arterial pressure (P_a) cerebrospinal fluid pressure (P_{CSF}) and perfusion pressure (P_p) in mm Hg

Lower part cortical blood flow (CBF_{grey}) in ml/100 g min and cerebrovascular resistance (CVR) in resistance units during the course of one experiment

A marked reduction of perfusion pressure resulting in a diminution of blood flow is followed by a pronounced reactive hyperemia lasting about one hour Two hours later a similar less marked reaction is seen

Fig 2 A 30 min period of perfusion pressure reduction in this case results in a hyperemia which is only partially restored after 70 min

creased during a stress period of about 90 min The arterial pressure showed a moderate Cushing effect The cerebral perfusion pressure was reduced as far down as to 20 mm Hg After this the cerebrospinal fluid pressure was reduced, bringing back the arterial and perfusion pressures to normal levels which were kept during the next 100 min, whereafter the experiment was repeated The lower curves show the cerebral cortical blood flow and the cerebrovascular resistance the latter being calculated as the ratio between the cerebral perfusion pressure and the cortical blood flow When the cerebral perfusion pressure was diminished stepwise the flow initially remained

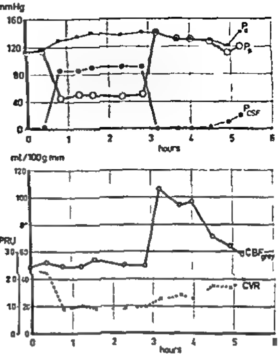

Fig 3 An example of a hyperemia elicited without noticeable previous diminution of blood flow

virtually unchanged, indicating a well functioning autoregulation However, when the perfusion pressure was reduced below 35 mm Hg a reduction in the cerebral blood flow took place After the reduction of the intracranial pressure with normalization of the arterial and perfusion pressures an increase in cerebral blood flow to values considerably higher than the control level ensued The increased cortical blood flow value returned to normal in the course of about 60 min The cerebrovascular resistance during the initial part of this hyperemia was about the same as at the end of the period of increased intracranial pressure As the hyperemia subsided the cerebrovascular resistance mounted to normal values When the experiment was repeated a similar reaction was observed, the hyperemia this time having a lower amplitude

Fig 2 shows an experiment with a perfusion pressure level of 38—42 mm Hg during a stress period of 30 min, thus considerably shorter than in the preceding experiment The hyperemia in this case had not subsided after an observation period of 70 min, though a tendency towards restoration to normal is obvious

The experiment depicted in Fig 3 demonstrates the hyperemia elicited after a 120 min period of cerebrospinal pressure increase Perfusion pressure was about 45—55 mm Hg during the stress period, a moderate Cushing effect was seen Although no reduction of the cerebral blood flow was observed, hyperemia ensued when the perfusion pressure was normalized In this case the hyperemia had virtually disappeared

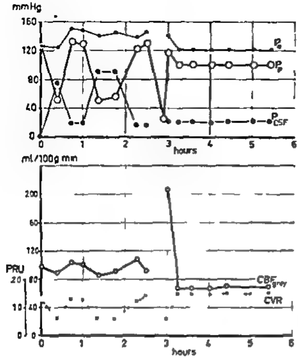

Fig 4 Two consecutive reductions of perfusion pressure to 50 mm Hg result in quite insignificant hyperemias but a marked hyperemia ensues after a pressure reduction to 25 mm Hg although this was of so short a duration that no flow measurement was possible

for a period of 120 min The cerebrovascular resistance showed the usual pronounced reduction implying a complete autoregulation during the stress period and returned slowly towards normal values during the period of restored perfusion pressure

Fig 4 demonstrates varying hyperemia responses to different induced cerebrospinal fluid pressure levels Whereas a perfusion pressure of 50 mm Hg on two occasions hardly induced any hyperemia responses the reduction of the perfusion pressure to 25 mm Hg gave a very pronounced short lasting hyperemia

Generally the hyperemic responses showed considerable variations in our material They were usually not observed unless the cerebrospinal fluid pressure was raised above 50 mm Hg corresponding to a perfusion pressure of about 70 80 mm Hg

The stress durations were usually between 15 and 120 min although in two cases shorter durations were used So in one animal with cerebrospinal fluid pressure of 130 mm Hg and perfusion pressure zero hyperemia was elicited after a stress time of 15 sec An important feature of the hyperemia phenomenon was its duration which was found to vary between 15 min—the usual time for a CBF measurement (see Discussion)—and periods exceeding 120 min In all cases where several measurements during one and the same hyperemic period were carried out the blood flow showed a tendency to return to normal though this tendency varied considerably In about half the experiments complete normalization was found the duration of the

hyperemic period in these cases varied between 15 and 120 min The longest hyperemia seen was observed for more than 3 hours and though slowly subsiding the flow did not reach the normal value during this period

The first blood flow measured during the hyperemic period was always the largest This maximum value could be 5 to 6 times the control flow

The flow during the stress period was measured in 20 out of 23 cases of induced hyperemia In 15 cases the flow was diminished before the hyperemia was elicited In 5 cases hyperemia was seen when no significant reduction of the blood flow had taken place The flow excess in these cases varied between 25 and 163 per cent of the control values All of them showed some reversibility 3 of them were completely normalized within the observation time

If the flow debt (the total integrated value of the underperfusion during the period of flow reduction) was considered in relation to the flow repay (the corresponding integral of the overperfusion during the hyperemia) it was found that the repay regularly exceeded the debt on an average by about 200 per cent The actual flow debt was usually repaid within 20 to 30 per cent of the stress duration time No correlation was found between on the one side the initial amplitude of the hyperemia and on the other the stress duration or the flow debt. The same was the case between the durations of stress and hyperemia Also the correlations between the amplitude of the hyperemia and the previous level of the raised cerebrospinal fluid pressure or between the duration of the hyperemia and the flow debt or perfusion pressure reduction were practically non existent

Special experiments were made in order to investigate the possibility of some chemical factor in the artificial cerebrospinal fluid being the cause of the hyperemic reaction Thus one experimental animal was subjected to a large craniotomy where by most of the bone frontoparietally over both hemispheres was removed The dura and arachnoid were incised in several places Thin polyethylene catheters were introduced in the basal cisterns and both lateral ventricles in order to facilitate the outflow from the CSF space The cerebrospinal cavity could thus be easily perfused with the artificial cerebrospinal fluid introduced through the double needle at pressures under 40 mm Hg *i e* lower than the minimum pressure required to elicit a detectable hyperemia However due to the diminished resistance to the outflow of the fluid the amount of fluid infused into the cisterna magna and passing the convexity of the hemispheres was 3—4 times the ordinary volumes Measurements of the cerebral blood flow for a period of 3 hours during this procedure showed virtually constant values

In another series of experiments different types of infusion fluid were tested In 3 animals reactive hyperemia could be produced by applying high cerebrospinal pressure with dextran, canine blood and human cerebrospinal fluid respectively Finally in one experiment canine cerebrospinal fluid was used also resulting in reactive hyperemia. It was therefore concluded that the hyperemia was caused by the induced pressure increase in the cerebrospinal cavity and not by any chemical property of the fluid used

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Concentration of Trypsin Inhibitors of Different Molecular Size and of Albumin and Haptoglobin in Blood and in Lymph of Various Organs in the Dog

By

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Abstract

GANROT P O, C-B LAURELL and K OHLSSON *Concentration of trypsin inhibitors of different molecular size and of albumin and haptoglobin in blood and in lymph of various organs in the dog* Acta physiol scand 1970 79 280—286

The concentration and homogeneity of some plasma proteins in lymph from various tissues in dog was studied immunochemically Special interest was focused on the enzyme inhibitors α_1 antitrypsin and α_2 macroglobulin The ratio between the concentrations in lymph and plasma was the same for albumin and α_1 antitrypsin but decreased significantly with increasing molecular size in the order α_1 antitrypsin, haptoglobin and α_2 macroglobulin The ratio α_1 antitrypsin/α_2-macroglobulin was on the average, twice as high in the intercellular fluid as in plasma The protein content of the lymph varied considerably between the organs with the highest values for lung and liver These organs also showed the lowest selectivity expressed as the quotient between the α_1-antitrypsin lymph/plasma ratio and the α_2 macroglobulin lymph/plasma ratio The influence of different rates of lymph flow in different organs on these values is discussed No enzyme inhibitor complexes were demonstrated

The distribution of various plasma proteins between the intravascular and extra vascular pools varies with their molecular size Thus in man less than half of the total amount of serum albumin is present in the blood compared with two thirds of the macroglobulin M (Jensen 1969) This indicates that the capillary walls are selectively permeable to proteins in the molecular weight range of 70 000 to 1 million. The permeability of the capillaries to proteins varies from tissue to tissue as reflected by the variation of the total protein concentration (1—5 g/100 ml) of lymph from different organs in the resting state (Rusznyak Foldi and Szabo 1969) This may be due to a variation in the filtration of fluid in relation to the restricted diffusion and leakage of proteins through the walls of the capillaries (Landis and Pappenheimer 1963, Majno 1965) According to some reports (Mayerson *et al* 1960 Taylor *et al* 1968) there is also a tissue to tissue variation of the molecular weight selectivity of the capillaries But most of these results have been obtained with exogenous sub-

stances The plasma proteins include enzymes, enzyme inhibitors and carrier proteins of different molecular sizes Estimation of the functional capacity of these proteins in the tissue fluid of various organs requires knowledge of the variation in the molecular size screening during passage through the capillaries The primary purpose of this study was to elucidate the distribution, in various tissues of the two inhibitors of proteolytic enzymes α_1 antitrypsin and α_2-macroglobulin with molecular weights of about 60 000 and 820 000 Two other plasma proteins albumin and haptoglobin (m w about 85,000) were used as references

Intracellular proteins and other substances may be released into the intercellular fluid and form complexes with extracellular proteins The lymph proteins might then differ in electrophoretic mobility and homogeneity from the corresponding plasma protein It was therefore desired to demonstrate the presence of complexes if any, between the enzyme inhibitors studied and proteolytic enzymes liberated or activated in the tissue

Material and methods

Experimental animals

12 healthy male and female mongrels, aged 1—3 years and weighing 15—20 kg were used

Anesthesia

Nembutal (Veterinary Nembutal Abbot 60 mg/ml) was used throughout A control sample (10 ml) of blood was obtained in tubes containing EDTA after which Nembutal was given i v in a dose of 30 mg/kg b w When necessary small supplementary doses were given to maintain light anesthesia All the dogs were intubated with a cuffed tube

Operative procedure

The operation was performed under aseptic conditions Lymph vessels in more than one area were cannulated in each animal The operation from the injection of the anesthetic until collection of the last lymph sample, never exceeded 2 hrs The operation involved practically no loss of blood

Cannulation of lymphatics

Polyethylene catheters PE 50 (inner diameter 0 58 mm) and PE 90 (inner diam 0 86 mm) were used

Leg lymphatics were cannulated in that segment running over the lateral aspect of the middle of the tibia together with the great saphenous vein The technique was largely that described by Grotte (1956)

a cannula was used

The thoracic duct was cannulated at the level of the neck in accordance with a method described by Markowitz (1949)

Lung lymph was obtained by direct cannulation of the right lymphatic trunk in one case and by the technique described by Leeds *et al* (1959) in the others An artificial chamber was formed from the internal and external jugular and subclavian veins in the area of the orifices of the right lymphatic channels Lymph was tapped from this chamber Only samples of lymph containing no chyle were accepted *i e* there were no large anastomoses between the thoracic duct and the right lymphatic duct Lymph from the head and neck was obtained by cannulation of the deep cervical lymphatics

TABLE I. The absolute values for lymph and serum albumin estimated by paper electrophoresis. The mean lymph albumin value and range for each organ is given together with the mean serum albumin value for the corresponding dogs

Lymph origin	Number of dogs	Lymph albumin g/100 ml	Serum albumin g/100 ml
Thoracic duct	7	3.44 (1.99—4.54)	4.44 (2.80—5.87)
Liver	4	3.94 (2.95—5.41)	4.54 (3.21—5.87)
Intestine	2	3.14 (2.50—3.77)	5.64 (5.41—5.87)
Leg	8	1.32 (0.81—2.85)	4.58 (2.80—5.87)
Head	3	2.60 (2.14—3.41)	4.07 (2.80—5.30)
Lung	4	3.12 (2.54—3.57)	4.44 (3.21—5.30)

Reagents

Agarose from L'Industrie Biologique Française S.A. Gennevilliers (Seine), France was used.

Antisera. Rabbit anti-α_1-antitrypsin, rabbit anti-α_2-macroglobulin, rabbit anti-haptoglobin were prepared by immunization with corresponding purified proteins from dog's plasma. Rabbit anti-human albumin cross-reacting with dog albumin was used.

Estimation of proteins

The relative concentrations of albumin, α_1-antitrypsin, haptoglobin and α_2-macroglobulin were determined by electrophoresis into agarose gel containing the corresponding antibody (Laurell 1966). A 0.075 M barbital buffer pH 8.6 was used and the potential gradient was about 20 V/cm. The concentrations were expressed relative to those in a reference pool of serum from healthy dogs. The serum albumin content was calculated from paper electrophoretic data.

Electrophoresis of sera

The electrophoresis was run in 1 mm thick cooled agarose gels (10° C) containing 0.075 M barbital buffer, pH 8.6 with 2 mM calcium lactate.

Electrophoretic homogeneity

The proteins were studied by antigen antibody crossed electrophoresis for homogeneity (Laurell 1965).

Results

Concentration of different proteins in lymph from various organs

The absolute concentrations of albumin in the serum samples and in the lymph samples from the same organ varied considerably from animal to animal (Table I). The lymph/plasma ratio of each of the plasma proteins in each lymph sample would therefore give better information on capillary protein passage than the absolute

TABLE II The lymph/plasma ratio of the proteins studied is given as the mean value and range for each organ and for the whole material

Lymph origin	Number of cases	Albumin	a_1-antitrypsin	Haptoglobin	a_2-Makroglobulin
Thoracic duct	7	0 76 (0 63—0 95)	0 73 (0 62—1 00)	0 62 (0 49—0 83)	0.28 (0 23—0.36)
Liver	4	0 86 (0 74—0 95)	0 85 (0 72—0 97)	0 48 (0 41—0.53)	0 51 (0 40—0 66)
Intestine	2	0 51 (0 45—0 56)	0.50 (0 46—0.53)	0 32 (0 23—0 41)	0 21 (0.20—0 22)
Leg	8	0 28 (0 16—0 43)	0 27 (0 17—0 39)	0 19 (0 12—0 29)	0 12 (0 07—0 17)
Head	3	0 68 (0 40—0 83)	0 62 (0 40—0 76)	0 22	0 19 (0 16—0.26)
Lung	4	0 71 (0 60—0 84)	0 70 (0 58—0 87)	0 63 (0 43—0 95)	0 44 (0.25—0 66)
All lymph samples	28	0 60 (0 16—0 95)	0 58 (0 17—1 00)	0 43* (0 12—0 95)	0 28 (0 07—0 66)

* only 26 lymph samples

values These ratios are summarized in Table II The ratios in the liver and lung lymph proved higher than in the intestine, head and leg In the thoracic duct, which receives lymph from various organs, the lymph had an intermediate ratio The lowest mean lymph/plasma ratio of all the proteins was found in samples from the leg

The individual lymph/plasma ratios of albumin and of a_1-antitrypsin were practically equal, while the ratio of haptoglobin was regularly lower, and that of a_2-macroglobulin still lower In the series of lymph samples as a whole, analysis of paired values with the t test showed the ratios of a_2-macroglobulin and haptoglobin to differ significantly ($P < 0.001$) from those of albumin The difference between the a_2-macroglobulin and haptoglobin ratios also proved significant ($P < 0.001$) But no such difference could be demonstrated between albumin and a_1 antitrypsin.

Capillary selectivity for proteins in various lymph regions

The relative permeability of the capillaries to the low molecular weight a_1-antitrypsin and to the high molecular weight a_2-macroglobulin was taken as a measure of the selectivity of the capillaries and it was expressed as the quotient between the a_1 antitrypsin lymph/plasma ratio and the a_2 macroglobulin lymph/plasma ratio The lowest selectivity values were found for the liver and lung (mean values 1 73 and 1 76 respectively) and the highest for the head (mean value 3.36) those for the intestine (mean 2 36) and leg (mean 2 24) lying in between The corresponding value for the thoracic duct lymph was 2 69 and that for all the samples 2 34

Electrophoretic mobility and homogeneity of lymph and plasma proteins

Judging from the results of antigen-antibody crossed electrophoresis the mobility and the degree of electrophoretic homogeneity of all four proteins studied were the same in plasma as in lymph. The method was sensitive enough to demonstrate whether more than 10 per cent of the molecules had been changed by complex formation. It was therefore possible to exclude any substantial complex formation or alteration by an attack of proteolytic enzymes.

Discussion

The ratio between the concentration of a protein in the lymph and in the plasma was taken as a measure of the permeability of the capillaries to the protein. But a high ratio does not necessarily mean that the capillary permeability to that protein (in the strict sense of the term) of a lymph region is high. It might also indicate that the capillary filtration of fluid in that region is low, as in the lungs (Yoffey and Courtice 1956, Courtice 1967, Rusznyak *et al.* 1969), with a more complete equilibration between intra- and extravascular fluids. It was not the aim of the present investigation to settle which of the two processes that mainly influenced the ratio, since the primary purpose was to study the relative concentration of trypsin inhibitors in lymph from different organs. The term "permeability to proteins" as used here therefore applies to all blood and lymph flow conditions contributing substantially to equilibration between the proteins of plasma and intercellular fluid. This terminology is in accord with that of Mayerson *et al.* (1960) and Taylor *et al.* (1968).

It has been shown that lymph capillaries are permeable to all known substances in the extracellular space (Courtice 1968, Yoffey *et al.* 1956, Rusznyak *et al.* 1969), while the permeability of the blood capillaries varies inversely with the molecular weights of the substances studied (Chien *et al.* 1964, Courtice 1968, Grotte 1956, Mayerson *et al.* 1960, Taylor *et al.* 1968, Wasserman, Loeb and Mayerson 1955, Yoffey *et al.* 1956, Rusznyak *et al.* 1969). Exogenous substances such as dextran, polyvinylpyrollidin and ^{131}I labelled albumin as well as the quotients between endogenous electrophoretic fractions or between albumin and globulin have been used in investigations of the permeability of capillaries. Fractions obtained by gel filtration of lymph and serum have also occasionally been compared (Bergstrom and Werner 1966). Poor agreement has been reported between the results obtained with endogenous and exogenous substances respectively. This has been explained by *inter alia* lack of accurate determination of the molecular weights of the protein fraction, different types of molecules in each fraction, incomplete equilibration of the exogenous substances between plasma and lymph even though the lymph/plasma ratio was apparently constant (Chien *et al.* 1964).

The quantitative immunochemical methods have paved the way for biologically more relevant and more specific studies and have been utilised by Taylor *et al.* (1968) for example in their investigation of the permeability of the capillaries in the

lung The enzyme inhibitors α_1-antitrypsin and α_2-macroglobulin are examples of extremely large and small plasma proteins and influence the activity of proteolytic enzymes in various respects

The protein content of lymph varies from one system to another Bone lymph and liver lymph have been regarded as examples of extremely high and low protein content Whether these two systems differ from one another regarding molecular screening also within the range of molecular size of protein is still debatable (Mayerson *et al* 1960 Taylor *et al* 1968) The data in Table II indicate that the capillaries in the liver and lungs are more permeable to large molecules than are the capillaries in the legs This finding is in agreement with Taylor's (1968) results, but not with the preliminary finding by Mayerson *et al* (1960) that the vessels emptying into the right duct were slightly less permeable than those in the neck But the high permeability to proteins and the low selectivity of the capillaries of liver and lung are probably not independent properties of these capillaries The maximal lymph/plasma ratio of a protein is probably about 1 0 (which does not mean that the capillaries are freely permeable to such a protein) If the lymph/plasma ratio of low molecular weight proteins is close to this maximal value, then a general increase of the permeability will necessarily influence the permeability to high molecular weight proteins more than to low molecular weight proteins and thus cause a decrease of the selectivity as it is defined here

The relative concentrations of α macroglobulin and α_1 antitrypsin indicate a selectivity in the capillary walls favouring α_1-antitrypsin in the intercellular space The ratio α_1 antitrypsin/α_2 macroglobulin was on the average twice as high in the intercellular fluid as in plasma This probably implies twice as high a relative linkage to α_1 antitrypsin of proteolytic enzymes like *e g* trypsin and elastase when released extravascularly than when released intravascularly This may be of special interest in the discussion of the fate of proteolytic enzymes released from the leucocytes succumbing in the peripheral tissues No enzyme inhibitor complexes were found on the immunochemical analysis of the lymph nor were any haptoglobin complexes found in the lymph as expression for any carrier function in the extracellular space It is however possible that complexes formed in the peripheral extracellular fluid are eliminated in the sieve of the lymph nodes

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References

BERGSTROM K and B WERNER Proteins in Human Thoracic Duct Lymph Studies on the distribution of some proteins between lymph and blood *Acta chir scand* 1966 *131* 413—422

CAIN J C J H GRINDLAY J L BOLLMAN E V FLOCK and F E MANN Lymph from Liver and Thoracic Duct *Surg Gynec Obstet* 1947 *85* 558—562

CHIEN S D G SINCLAIR C CHANG B PERIC and R J DELLENBACK Simultaneous study of capillary permeability to several macromolecules *Amer J Physiol* 1964 *207* 513—517

COURTICE F C Lymph formation in the lungs *Jap Heart J* 1967 *8* 729—733

COURTICE, F C, Lymphatic function Concepts old and new *The Medical Journal of Australia.* 1968 9 379—385
DE ANDRADE, V, E M BOZYMSKI and J T SESSIONS JR., A method for cannulating mesenteric lymphatic vessels in dogs *J Lab clin Med* 1968 *72* 521—526
GROTTE, G, Passage of dextran molecules across the blood lymph barrier *Acta chir scand* Suppl *211* 1956
JENSEN, K B, Metabolism of Human γ Macroglobulin (IgM) in normal man *Scand J clin Lab Invest* In press
LANDIS E M and J R. PAPPENHEIMER Exchange of substances through the capillary walls. In *Handbook of Physiology* section 2 Circulation, vol *II* Ed W F Hamilton American Physiol Soc, Washington, D C 1963
LAURELL, C B, Antigen antibody crossed electrophoresis *Analyt Biochem* 1965 *10* 358—361
LAURELL, C. B, Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies *Analyt Biochem* 1966 *15* 45—52
LEEDS, W E, H N UHLEY J J SAMPSON and M FRIEDMAN A new method for measurement of lymph flow from the right duct in the dog *Amer J Surg* 1959 *98* 211—216
MAJNO G, Ultrastructure of the vascular membrane In *Handbook of Physiology,* section 2 Circulation, vol *III* Ed. W F Hamilton American Physiol Soc, Washington D C 1965
MARKOWITZ, J, *Experimental Surgery,* The Williams and Wilkins Company, 1949 Baltimore
MAYERSON H S C G WOLFRAM, H H SHIRLEY, JR and K WASSERMAN Regional differences in capillary permeability *Amer J Physiol* 1960 *198* 155—160
RUSZNYAK, I, M FOLDI and G SZABO *Lymphologie, Physiologie und Pathologie der Lymphgefasse und des Lymphkreislaufes* Gustav Fisher Verlag Stuttgart 1969
TAYLOR P M, U BOONYAPRAKOB, D W WATSON and P FIREMAN Relative efflux of native proteins from the canine pulmonary vascular bed *Amer J Physiol* 1968 *214* 1310—1314
WASSERMAN K. L. LOEB and H S MAYERSON, Capillary Permeability to Micro-Molecules. *Circulat Res* 1955 *3* 594—603
YOFFEY, J M and F C COURTICE, *Lymphatics Lymph and Lymphoid Tissue* Edward Arnold (Publ) Ltd 1956

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Release of a Smooth-Muscle-Active Substance besides Noradrenaline from Degenerating Sympathetic Terminals in the Rabbit Eye

By

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In connection with studies concerning the early effects of sympathetic denervation on the rabbit eye we have made some observations which we believe to be of interest to non-ophthalmologists They are summarized below Full reports with references are in the process of being published elsewhere (Treister and Bárány 1970 a, b, c)

14 to 19 hrs after removal of the superior cervical ganglion in the albino rabbit 3 transient intraocular phenomena start a dilatation of the pupil (mydriasis), a hyperemia of the iris vessels and a lowering of the intraocular pressure (at least partly due to a reduction in the resistance to the outflow of aqueous humour through the chamber angle) The time relations of these phenomena in the conscious undrugged animal are shown in the upper panel of Fig 1 If 10 mg/kg bretylium is given at 0 and 10 hrs, the time relations become those of the lower panel Thus, bretylium delays the mydriasis by about 5 hrs but the hyperemia and the pressure drop by about 9 hrs

While the mydriasis at first seemed to be a simple instance of degeneration contraction of the iris dilatator muscle, it turned out to be more complex Only during the first few hours is it fully reversible by phentolamine or preventable by phenoxybenzamine or reserpine The later parts, starting about 17 hrs after denervation, contain a component resistant to these drugs The hyperemia too is not preventable by phenoxybenzamine or reserpine and not affected by phentolamine, propranolol, spiropendol chlorpromazine, butoxamine lidocain or atropine The pharmacology of the pressure drop has not been studied directly but the decrease in outflow resistance is also very resistant to a variety of anti adrenergic drugs.

Our observations indicate that besides noradrenaline another smooth muscle active substance appears when sympathetic nerve terminals degenerate This substance appears only after the degeneration release of noradrenaline is well under way Bretylium delays the appearance of this substance much more than that of noradrenaline Reserpine does not prevent its appearance It is therefore improbable that the substance is released by the noradrenaline leaking out of the terminals

[1] Work done during leave of absence from Tel Hashomer Hospital Tel Aviv University, Medical School Israel (Dr Treister)

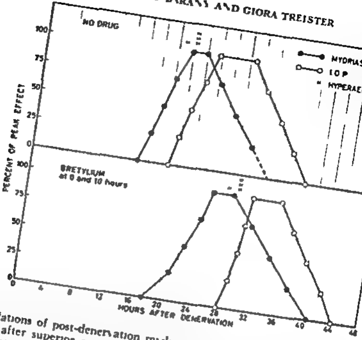

Fig 1 Time relations of post-denervation mydriasis, intraocular pressure drop (IOP) and r hyperemia (H) after superior cervical ganglionectomy in albino rabbits
The effects were measured or estimated by comparison with the contralateral eye, where preganglionic sympathotomy) had been performed H indicates the appearance of the first def inite hyperemia H full fledged hyperemia
H

The nature of the substance is still unknown Whether it is specific for the rabb ve or if the rabbit eye is especially sensitive to it remains to be investigated In th rabbit ear no corresponding hyperemia is seen (Bárány and Treister 1970 d)

Supported by grants EY-00231 from the National Eye Institute U S Public Health Service and grant K69 14X 733-04C from the Swedish Medical Research Council

References

RANY E H and G TREISTER Time relations of degeneration mydriasis and degeneration vasoconstriction in the rabbit ear after sympathetic denervation Effect of bretylium Acta physiol scand 1970d In press
EISTER G and E H BÁRÁNY Mydriasis and intraocular pressure decrease in the conscious rabbit after unilateral superior cervical ganglionectomy Invest Ophthal 1970 a. In press
EISTER G and F H BÁRÁNY The effect of bretylium on the degeneration mydriasis and intraocular pressure decrease in the conscious rabbit after unilateral cervical ganglionectomy vest Ophthal 1970 b In press
STER G and E H BÁRÁNY Degeneration mydriasis and hyperemia of the iris after perior cervical ganglionectomy in the rabbit Invest Ophthal 1970 c In press

Abstracts from Meeting of the Scandinavian Physiological Society in Helsinki 24—25 April 1970.

DEMONSTRATIONS

D 1

Department of Physiology, University of Göteborg Sweden

Effects of Vasoconstriction on Blood Viscosity in Vivo

By

P. Baeckström, B. Folkow, E. Kendrick, B. Löfving and B. Öberg

At high flow rates in maximally dilated skeletal muscle vessels of cats, blood viscosity *in vivo* has been shown to be only about half of that *in vitro* at high shear rates, increasing almost two times with flow reductions and decreasing again at the very lowest flow rates (Djojosugito *et al.* 1970). This is in contrast to the marked increase of blood viscosity *in vitro* at low shear rates. The reduction of blood viscosity at extremely low flows in the completely relaxed vascular bed seems to be dependent on the concomitant passive recoil and narrowing of the vessels, favouring the emergence of bolus flow and the Fåhraeus Lindquist effect in those vascular sections where the major flow resistance resides (Folkow *et al.* 1969).

The present experiments were undertaken to analyse whether a vessel narrowing produced by intense vasoconstriction, with a flow resistance 25—50 times higher than during maximal dilatation, has a similar viscosity-reducing effect. The isolated calf muscle vascular bed of the cat was used as a biological viscometer. Comparisons of the pressure flow curves for blood and a Newtonian fluid (dextran Tyrode) were made, both with the vessels maximally dilated (exercise + i.a. injection of dilator drugs) and when they were brought into a stable and intense constriction by means of barium ions.

At identical very low flows blood viscosity was decidedly lower in the intensely constricted vascular bed than in the maximally dilated bed and appeared to be only about 30 per cent higher than that found at maximal flows. These data indicate that the wide range of flows, met with *in vivo* as a result of alterations in vascular tone, is associated with only moderate changes of effective blood viscosity. Therefore, in the *in vivo* situation blood viscosity does not seem to be an important determinant of blood flow in a constricted vascular bed, not even at the very low flows met with in shock, provided that blood *composition* has not been concomitantly altered.

References

DJOJOSUGITO A M B FOLKOW B ÖBERG and S WHITE *Acta physiol scand* 19 0 78 70—84
FOLKOW B B LOFVING and B ÖBERG *Acta physiol scand* 1969 suppl 330 p 90

D 2

Departments of Physiological Chemistry and Anatomy University of Umeå Sweden

Gastric Digestion and Uptake of Milk Fat in the Suckling Rat

By

T EGELRUD H F HELANDER and T OLIVECRONA

A lipase is secreted by the gastric mucosa but is probably of minor importance for the gastric digestion in the adult since its pH optimum is between 5 and 7 The present paper demonstrates that in suckling rats whose gastric pH is close to neutrality significant quantities of lipids are hydrolyzed and absorbed in the stomach

In fresh rat milk triglycerides made up 85 % of the total lipids whereas partial glycerides free fatty acids and phospholipids were present only in small quantities About 1/4 of the triglyceride fatty acids were of medium chain length the rest being of long chain length In the gastric contents of suckling 10 day old rats about 50 % of the triglycerides had been hydrolyzed to long chain diglycerides and medium chain free fatty acids Thus in these rats gastric lipolysis is of quantitative significance

By light and electron microscopy the gastric mucosa was studied in suckling and fasted infant rats Large lipid droplets up to 5 μ in size were observed in surface epithelial cells of the non fasted animals but were absent in the fasted ones The lipid droplets were lying free in the cytoplasm and were not attached to any membrane This is in contrast to the intestinal epithelium where absorbed lipids appear as droplets surrounded by a smooth surfaced membrane—possibly a factor of significance for the re esterification of absorbed glycerides When fasted infant rats were given a mixture of milk and peroxidase this enzyme could later be demonstrated in the cytoplasmic lipid droplets For this reason it seems probable that part of the absorption is accomplished by pinocytosis No lipid material was observed in the intercellular space or in the capillaries indicating that bulk transport of lipids into the circulatory system is limited

Biochemical analyses showed that gastric walls from suckling rats contained partial glycerides and free fatty acids in proportions similar to those in the gastric contents In gastric walls from fasted rats these lipids were present in considerably smaller quantities Studies on the metabolism of various C^{14} labeled lipids in the stomach of infant rats indicated that only free fatty acids were transferred in significant quantities into the circulatory system

D 3

Department of Physiological Zoology and Department of Genetics University of Helsinki, Finland

Composition of Alcohol Dehydrogenase Isoenzymes in Drosophila subobscura

By

S LAKOVAARA and A SAURA

There is general agreement that the multiple forms of an enzyme within an organism are caused by association of active subunits to form enzyme molecules of different molecular structure *Drosophila melanogaster* alcohol dehydrogenase (ADH) has been previously assumed to be composed of subunits (Johnson and Denniston 1964), but there is more recent evidence for NAD as a factor responsible for appearance of ADH isoenzymes (Jacobson 1968 and Ursprung and Carlin 1968)

We have described an ADH polymorphism analogous with that of *D melanogaster* in *D subobscura* by starch gel electrophoresis (Lakovaara and Saura 1970) The results are illustrated in Fig 1 The marginal types a and c are ADH homozygotes and b is the heterozygote of these

The distal bands a and b 5 and 6 and c 4 and 3 are denatured at 45° C the proximal bands being stable at this temperature When incubated at +2° C only the isoenzymes sensitive to heat show ADH activity An addition of 50 mg NAD per 50 ml gel causes a contraction of the isoenzyme pattern and a dislocation of the distal bands 6 and 5 to position 4 in types a and b and in c 4 is shifted to position 3 Storage for several months does not affect very much the total ADH activity but causes a strenghtening of the minor bands 5 and 3 in homozygotes resulting into a hybrid appearance

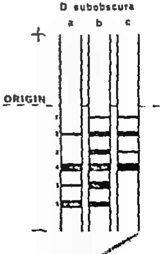

Fig 1 For description see text

The demonstration that the ADH isoenzymes differ in their coenzyme content does not rule out polymerization as a cause for the multiple forms observed in electrophoresis. The bands 5 and 3 in the heterozygote can in our opinion best be explained as representing association products of certain subunits that are active as such. Because bands 5 and 6 do not appear in a after prolonged storage, this seems to indicate that there exist two alleles of a single gene, a and c that produce the postulated subunits. This has been demonstrated to be the case by crossing experiments.

References

JACOBSON, K. B.: *Science* 1968, *159*, 324—325.
JOHNSON, F. M. and C. DENNISTON: *Nature* 1964, *204*, 906—907.
LAKOVAARA, S. and A. SAURA: *Ann. Acad. Sci. fenn. A, II Biologica* 1970, *163*, 1—10.
URSPRUNG, H. and L. CARLIN: *Ann. N. Y. Acad. Sci.* 1968, *151*, 465—475.

D 4

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Intracellular Staining of Physiologically Identified Interneurones in the Cat Spinal Cord

By

E. JANKOWSKA and S. LINDSTRÖM

Interneurones in the spinal cord may be physiologically defined by the connexions to them from primary afferents or descending pathways. In order to identify a recorded interneurone as belonging to a certain neuronal pathway it is desirable to know its axonal projection. The new technique of iontophoretic injection of a fluorescent dye (Procion Yellow, ICI) into the cells elaborated by Stretton and Kravitz (1968) in crustacea, allows morphological investigation of cells from which the intracellular recording has been made.

This technique has now been used for staining different types of interneurones in the spinal cord. In each interneurone intracellular records of postsynaptic potentials from different groups of afferents and descending tracts were taken, thus defining its input. The microelectrodes used for recording were filled with a 5 % solution of the dye, which was injected into the cells by constant current of 10—20 nA during 10—15 min. The staining technique was previously adjusted for cat spinal cord neurones (Jankowska and Lindström 1970). After histological procedures the trajectory of dendrites and axons of the stained cells could be reconstructed from serial sections as exemplified in the figure. The illustrated cell was polysynaptically activated from

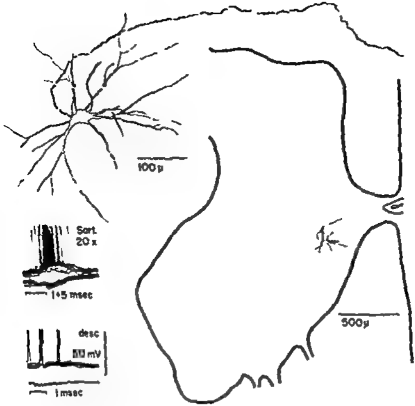

Fig 1 Reconstruction of soma-dendrites and axon (filled) of an interneurone stained with Procion Yellow Tracings from 20 sections 15 µ thick The records show intracellular potentials recorded in the same neurone evoked from sartorius nerve and descending axons

ipsilateral high threshold muscle and cutaneous afferents and monosynaptically from ipsilateral descending axons The dendritic tree and the axons of the injected cells could be seen over a distance from the soma of about 400 µ and 900 µ respectively

It is thus possible to establish the direction of the axonal projection of a physiologically identified interneurone It is hoped that with improved technique also the fine axonal branches and terminals may be identified or else that the intracellular staining technique may be combined with conventional histological methods

References

JANKOWSKA E and S LINDSTROM *Brain Research* 1970 submitted for publication
STRETTON O W and E A KRAVITZ *Science* 1968 *162* 132—134

D 5

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The Stability of the Metabolic Gradient in the Rat Small Intestine

By

A Aitio, O Hänninen and E Hietanen

There is a marked metabolic gradient in the rat small intestine as indicated by the decrease of O_2-uptake, CO_2-production and the activities of several enzymes of the phosphate and glucuronate metabolism (e g Aitio and Hanninen 1968 Hanninen, Aitio and Hartiala 1968) In order to study to what extent this gradient is due to the specialization of the cells and loss of adaptability we have changed the normal chyme passage by gastrointestinal surgery given perorally an inducing agent cinchophen and compared the specific pathogen free (SPF) and conventional rats in total 300 rats were used

After partial jejunectomy and gastrojejunostomy with pyloric ligation there was a marked mucosal hypertrophy in the operation area which declined slowly with time The mucosal fresh weight was also higher in jejunum of the conventional than SPF rats Despite of hypertrophy the activity of alkaline phosphatase remained in mucosa unchanged for several weeks in partially jejunectomized rats Also in gastrojejunostomized rats its activity remained unchanged long but in 2 5 months there was a slight increase in the operation area The ATPase activity decreased after partial jejunectomy in two months In gastrojejunostomized rats there was a decrease orally but an increase aborally from the gastrojejunostomy Maltase activity increased slowly in partially jejunectomized rats in the aboral gut In gastrojejunostomized rats its activity was depressed in the operation area o-Aminophenyl glucuronide synthesis was increased in the gastrojejunostomy area but decreased in 2 5 months back to the control level No changes were found in partially jejunectomized rats In isolated jejunal loops the mucosa atrophied to a great extent the alkaline phosphatase and glucuronide synthesizing activity decreased but the maltase activity remained unchanged

In the SPF rats the activity of alkaline phosphatase ATPase and maltase were higher than in the conventional rats and so was also the UDP glucuronyltransferase activity in the duodenum

The administration of cinchophen caused an increase of glucuronide synthesis in the whole gastrointestinal tract The gastrojejunostomized rats were very sensitive to the drug but in those rats who survived the treatment showed a similar response as unoperated rats

The data obtained indicate that the metabolic gradient in the rat small intestine is remarkably stable in the changes of chyme flow although the mucosa hypertrophies

rapidly Most changes of enzyme activities are rather small and they appear slowly The glucuronide synthesis is however, sensitive to changes in chyme flow and cinchophen given perorally The stability is a remarkable fact, since the renewal time of mucosal cells is only two days The cells in the different areas of the small intestine are probably highly specialized although they resemble each other morphologically

References

AITIO A and O HANNINEN *Acta physiol scand* 1968 74 13A
HANNINEN O A AITIO and K HARTIALA *Scand J Gastroenterology* 1968 3 461

D 6

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Techniques for Single Unit Recording in Behaving Monkeys

By

J HYVARINEN

For combining in a single experiment behavioral and electrophysiological recording the following routine is developed. Monkeys *(Macaca speciosa)* are trained to sit in a chair and to press a lever when a sinusoidal mechanical vibration is applied on the skin of the palm (Mountcastle *et al* 1969) Prior to recording sessions a metal halo ring is screwed to the skull for immobilization of the head and a metal cylinder is implanted over the postcentral gyrus A hydraulic microdrive is used for the advancement of glass coated platinum microelectrodes Wilska 1940) through the intact dura mater (Evarts 1968) into the primary hand area of the somesthetic cortex while the vibrator is placed on the receptive field of the cells encountered and the monkeys are performing their task These techniques allow study of spontaneous and driven single unit activity under various physiological conditions without suppressing action of any drugs

References

EVARTS E. V *Electroenceph clin Neurophysiol* 1968 24 83—86
MOUNTCASTLE V B W H TALBOT H SAKATA and J HYVARINEN *J Neurophysiol* 1969 32 452—484
WILSKA A *Duodecim (Helsinki)* 1940 72 63—75

D 7

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The Termination of the Cervicothalamic Tract in the Cat

By

J Boivie

The fastest pathway for cutaneous impulses from the trunk and limbs to the thalamus or the cerebral cortex is not *via* the dorsal columns but *via* the spinocervicothalamic pathway (SCTP) (Landgren *et al* 1965, Andersson 1962) After a synaptic relay in the ipsilateral cervical nucleus (LCN) the pathway crosses the midline to ascend in the contralateral medial lemniscus (ML) to the thalamus With neurophysiological methods Landgren *et al* (1965) mapped the area of termination of the SCTP in the thalamus They found that it was restricted to the rostral pole and the dorsal lateral and ventrolateral aspects of the nucleus ventralis posterolateralis (VPL)

Following LCN lesions of various extent the degenerating fibres in the thalamus were mapped light microscopically in Nauta and Fink Heimer stained sections (Fig 1) The results make it highly probable that the LCN axons terminate in the following thalamic structures the lateral and medial parts of the VPL (VPLl and VPLm respectively) the medial part of the posterior nuclear complex (POm) and the magnocellular part of the medial geniculate nucleus (MGmc) The terminal degeneration seemed to be most abundant in the dorsal part of the VPLl dorsolaterally in the rostral third of the VPLm and in the MGmc which contained degeneration only in its most medial part In the POm the degeneration was scattered The lateral two thirds of the nucleus ventralis posterior inferior (VPI) also contained degenerating fibres but these were probably passing and not terminal ones

The distribution of the degenerating fibres was the same regardless of whether the lesions were restricted to the rostral or the caudal parts of the LCN and did not differ fundamentally from that in cats with complete LCN destructions

References

ANDERSSON S A *Acta physiol scand* 1962 56 Suppl 194 1 74
LANDGREN S A NORDWALL and C WENGSTRÖM *Acta physiol scand* 1965 65 1[illegible]
RINVIK E *Brain Res* 1968 8 23 [illegible]54

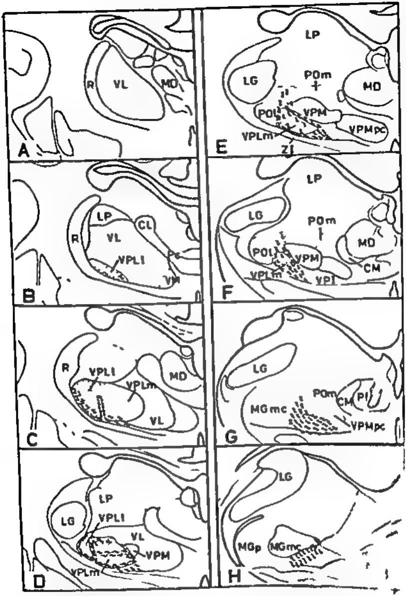

Fig 1 Drawings of representative transverse sections of the cat [illegible] 1968 showing the distribution of degenerating terminal and [illegible] and wavy lines respectively) in the contralateral thalamus [illegible] whole LGN Intervals between drawings about 650 μ

D 8

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^{133}Xe Clearance from Musculus Quadriceps Femoris during Concentric and Eccentric Bicycle Exercise at Different Temperatures and Loads

By

F. Bonde Petersen, B. Nielsen, S. Levin Nielsen and L. Vanggaard

The effects of muscle tension and ambient temperature upon muscle blood flow during exercise was investigated. A modification of Krogh's bicycle ergometer (Bonde Petersen 1969) was employed for positive and negative work. A work load of 1260 kpm/min was chosen for negative work and three positive work loads (252, 756 and 1260 kpm/min) were chosen which gave similar oxygen consumption, heat production, and muscle tension, respectively. Muscle blood flow was calculated from ^{133}Xe clearance (m. vastus lateralis). Cardiac output (CO_2 and acetylene methods) was also measured.

Muscle blood flow was directly related to oxygen consumption. There was a levelling-off of blood flow in spite of increasing work load. This has previously been observed by Tonnesen (1964) and Clausen and Lassen (1970) and has been attributed to increasing intramuscular pressure during contraction at higher loads.

However, muscle tension was not found to reduce muscle blood flow in the present experiments, as similar flow values were obtained in positive (252 kpm/min) and negative (1260 kpm/min) work at the same oxygen consumption. The tensions in the negative work are obviously much greater.

The levelling-off of muscle blood flow in spite of increasing work loads is explained by an increase in the oxygen extraction the closer the muscle are working to their maximum work load. Ambient temperature (20—35° C) was without influence upon oxygen consumption or ^{133}Xe clearance.

References

Bonde Petersen, F.: Int. Z. angew. Physiol. 1969, 27, 133—137.
Clausen, J. P. and N. A. Lassen: Nordisk med. 1970, 83, 94.
Tonnesen, K. H.: Scand. J. clin. lab. invest. 1964, 16, 646—654.

C 1

Department of Physiology I, Karolinska Institutet Stockholm Sweden

Ultrastructural and Biochemical Studies of Purified Noradrenaline Storing Nerve Trunk Vesicles

By

A KLEIN, R L KLEIN, H LAGERCRANTZ and L STJÄRNE

In order to study the enzymatic properties and the uptake and storage of nor adrenaline (NA) in isolated storage vesicles from bovine splenic nerve trunk it is desirable to have preparations relatively free of enzymes belonging to other cell com ponents or at least to have knowledge of the activity of contaminating enzymes

By using a special technique with heavy water sucrose gradients we have obtained a NA vesicle fraction essentially free of microsomal marker enzyme activity In addi tion mitochondrial lysosomal and Golgi markers are substantially reduced relative to the increase in NA protein ratio

Three fractions were obtained after ultracentrifugation (see Fig 1) The lowest one (F III) contained an average of 3 44 μg NA/mg protein which represents a 50 fold purification of particle bound NA after homogenization of whole splenic nerves and a 4—7 fold purification over values reported in the literature Fractions F II and F III together contain 55 per cent of the sedimentable NA at 2 7 μg NA/mg protein

By considering the percentage distribution of NA protein and the average contam ination of the marker enzymes in the various fractions one can calculate that a minimum of 18 per cent of F III is NA vesicle protein If the vesicles were 100 per cent pure the upper limit of NA protein could be estimated at 28 7 μg NA/mg protein Electron microscopic examination of F III shows morphological differences between Mg^{++} ATP treated and NA depleted vesicles It also indicates that the purity could conservatively be estimated at 40—50 per cent on a volume basis Chromogranin analyses give further support (Karen Helle pers commun)

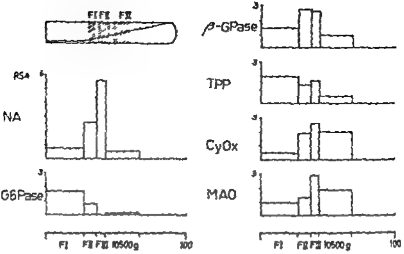

Fig 1 Splen [illegible]

abscissa per cent of total protein Means of 6 experiments

C 2

Institute of Anatomy and Histology, University of Lund, Sweden

Monoamine-Like Substances in the Pituitary Gland

By

A Bjorklund and A Nobin

By using recently developed modifications of the Falck-Hillarp fluorescence method (Bjorklund, Falck and Håkanson 1968) two fluorogenic monoamine-like substances have been found in the anterior and intermediate lobes of the pituitary gland One of these substances which is closely related to, or possibly identical with, tryptamine is localized in the cells of the intermediate lobe as well as in a population of cells in the anterior lobe The cells in the anterior lobe have been suggested to be identical with the ACTH producing cells

The second fluorogenic substance is localized in PAS positive cells in the anterior lobe The fluorophore formed from this substance upon formaldehyde treatment showed photodecomposition and quenching characteristics in common with the fluorophores of the biogenic catecholamines The fluorophore formed from the substance stored in PAS-positive cells exhibited a pH-dependent fluorescence intensity, the visible fluorescence being very weak at neutral or alkaline pH s and strong at

acid pH The fluorophore showed characteristic and reproducible spectra, with excitation and emission maxima at 350 and 465 mμ in acidified sections The characteristics of this fluorophore are different from those of the fluorophores of the biogenic catecholamines, 5 HT, tryptamine and their precursor amino acids, and suggest that the compound stored in the PAS-positive cells is a phenyl ethylamine, hitherto not identified Furthermore, L dopa (but not D-dopa or dopamine) was taken up into these cells and probably decarboxylated to dopamine In what way the exogenous L-dopa influences the endogenous fluorogenic substance will be the subject of further investigation

The inter individual variation in number and intensity of the fluorescent cells was most conspicuous, and this variation could mainly be ascribed to a difference between the two sexes Thus, in the male rat and mouse a large number of moderate to strong fluorescent cells occurred in contrast to the females where a considerably smaller number of fluorescent cells with a generally lower intensity was found

After endocrine manipulations including castration, thyroidectomy and substitution therapy characteristic changes have been found in the content of the monoamine-like substance in the PAS positive cells Since PAS positive cells are known to be the site of production of gonadotrophic and thyrotrophic hormones these findings may indicate a functional significance for the fluorogenic substance in the cells

References

BJORKLUND, A, B FALCK and H HÅKANSSON *Acta physiol scand* 1968, suppl 318 1—31

C 3

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Is the Tonic Stretch Reflex Dependent on Suppression of Autogenetic Inhibitory Reflexes?

By

S GRILLNER and M UDO

The tonic stretch reflex occurs together with a high level of activity in static γ motoneurones (Grillner 1969) In the decerebrate cat descending pathways in the ventral quadrant and in particular the vestibulospinal tract has been held responsible (*e g* Sprague and Chambers 1953) this pathway has monosynaptic excitatory connexions to α motoneurones and static γ motoneurones of hindlimb extensors (Grillner 1969)

In the present investigation however lesions to the ipsi or the contralateral dorsolateral funicle (Th 13 or C2) or medial lesions at the level of the obex have been found to reduce the gain (expressed as increase in total tension minus passive

tension with an extension of 1 mm) of the stretch reflex (soleus or triceps surae in the inter-collicularly decerebrated cat) to between 50 and 10 % of the original value, the threshold for initiation of the stretch reflex on the other hand appeared less influenced After these lesions it was shown that the vestibulospinal tract was intact by means of antidromic stimulation of the fibres caudal to the lesion and recording of the antidromic field potential in the Deiters nucleus (cf Grillner 1969) Hence it can be concluded that the tonic stretch reflex is dependent not only upon the vestibulospinal tract but also on another system

The different lesions at remove tonic inhibition from descending fibres of inhibitory reflex arcs to extensor motoneurones (Holmqvist and Lundberg 1959), thus there will be a release of the transmission in the autogenetic inhibitory pathways from group Ib and II afferents (cf Grillner 1970) Although the effect of the lesion on the discharge in , motoneurones is not known, it is possible that the increased effectiveness of the inhibitory reflex arcs activated during stretch of the muscle overrides or decreases the excitatory effect from group Ia afferents It should be noted that in the three acute preparations in which a tonic stretch reflex can be elicited (decerebrate cat, spinal cat after an intravenous injection of DOPA Grillner 1969 spinal cat after an injection of 5 HTP, Ahlman Grillner and Udo unpublished) there is a suppressed transmission in the reflex arcs from group II and other afferents

The above considerations allow the tentative suggestion that not only a high level of static fusimotor activity is required in order to obtain a tonic stretch reflex but also a suppression of the autogenetic inhibitory reflex arcs

References

GRILLNER S *Acta physiol scand* 1969 Suppl 327 1—34
GRILLNER S *Acta physiol scand* 1970 *78* 431—432
HOLMQVIST B and A LUNDBERG *Arch ital Biol* 1959 *97* 340—356
SPRAGUE J M and W W CHAMBERS *J Neurophysiol* 1953 *16* 451—463

C 4

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IPSPs in the Slowly Adapting Stretch Receptor of the Crayfish

By

J K S JANSEN A NJÅ K ORMSTAD and L WALLOE

The large accessory fibre of the abdominal stretch receptors of the crayfish has long been known to be inhibitory (Kuffler and Eyzaguirre 1955) On indirect evidence it has been suggested that there is a second inhibitory efferent nerve fibre innervating these receptors (Burgen and Kuffler 1957 Jansen Njå and Walloe 1969)

We have now recorded the electrical activity of the slowly adapting receptor intra

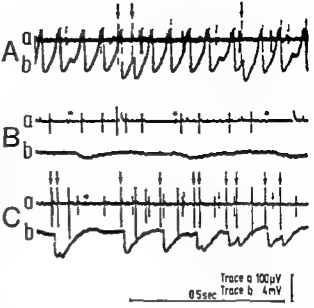

Fig 1 Records of membrane potential of a slowly adapting receptor and of the activity of the corresponding superficial nerve A During receptor firing Trace *a* nerve activity Trace *b* receptor membrane potential Large and small accessory spikes marked with ↓ ↓ respectively Note the difference in size of their IPSPs The receptor action potentials are too faint to reproduce in record from nerve and barely visible in intracellular record B Same receptor and same type of records as A obtained during period of 'spontaneous' activity Small action potential in nerve associated with IPSPs of the receptor indicated by * C Same receptor All three types of IPSPs present during reflex activation from stretch receptor of neighbouring segment Each nerve spike marked by the appropriate symbol

cellularly during various reflexes Three different types of IPSPs have been observed in the receptor They are all associated with different efferent action potentials in the superficial nerve innervating the receptor (Fig 1) Two of the IPSPs can be reflexly activated by the same or neighbouring receptors Their axon spikes have greatly different amplitudes and the latencies of the corresponding IPSPs suggest axonal conduction velocities of approximately 2 m/sec for the largest and 1 m/sec for the smallest These two spikes presumably belong to the large and the small accessory axons of Alexandrowicz (1967) The large spike evokes a large IPSP with rapid time course The small spike evokes a small and slower IPSP The third IPSP is still smaller and is associated with the smallest axonal spike It has so far not been reflexly excited but occurs periodically as a 'spontaneous' inhibitory background activity The conduction velocity of its axon is approximately 0.5 m/sec

The inhibitory nature of these IPSPs is shown by their ability to delay receptor discharges Their mode of action is presumably by an inhibitory increase in conductance since their amplitudes are directly dependent of the level of the membrane potential of the receptor

We conclude that the slowly adapting stretch receptor of the crayfish abdomen is innervated by at least three inhibitory axons The smallest of these may be the fiber described anatomically and designated x by Alexandrowicz (1967)

References

Alexandrowicz J S *Biol Revs* 1967 *42* 288—326
Burgen A S V and S W Kuffler *Nature* Lond 1957 *180* 1490—1491
Jansen J K S A Njå and L Walløe *Acta physiol scand* 1969 *76* 29A—30A
Kuffler S W and C Eyzaguirre *J gen Physiol* 1955 *39* 155—184

C 5

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Capillary Density of Skeletal Muscle in Well-trained and Untrained Men

By

L Hermansen and M Wachtlova[1]

Capillary density was studied in biopsy samples from the lateral part of m quadriceps femoris in 15 young males, 8 untrained (maximal oxygen uptake 50 2 ml/kg×min) and 7 well-trained (maximal uptake 71 4 ml/kg×min) subjects

The numbers of capillaries per mm² was found to be $611 \pm 28\ 3$ (mean ± SE) and $599 \pm 26\ 6$ in the well trained and untrained subjects respectively The difference was not statistically significant The number of muscle fibres per mm² was significantly lower in the well trained, than in the untrained group, and the capillary/fibre ratio was 49 9 per cent lower in untrained than in well trained subjects

There was, however, no significant difference in the diffusion distance (average half distance between two capillaries) between the two groups

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C 6

From the University Laboratory of Physiology Oxford England

Changes in the Sensitivity of the Baroreflex in Muscular Exercise

By

D J C Cunningham M G Howson E Strange Petersen[1] T G Pickering and P Sleight

The aim of this study was to apply a quantitative method of testing baroreflex sensitivity (Smyth, Sleight and Pickering 1969) to the control of heart rate during exercise

Reflex cardiac slowing was produced by a transient rise of arterial pressure induced by an intravenous injection of phenylephrine (50—180 μg) When pulse in

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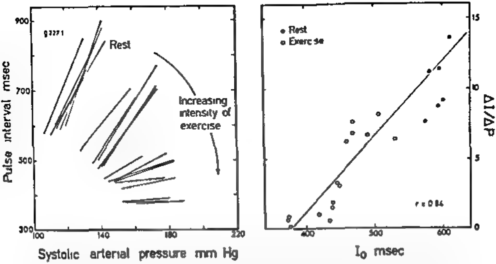

Fig 1 All results of an experiment on one subject In the left panel each individual line represents an injection of phenylephrine The starting points of the lines indicate pulse interval and systolic pressure in the various steady states prior to the drug induced pressure rises The

terval (I) is plotted against systolic pressure (P) a linear relation is obtained, the slope of which (msec increase of I/mm Hg rise in P) is used as an index of baroreflex sensitivity, the steeper the slope the greater the sensitivity

3 or 4 injections of phenylephrine were given to four subjects at rest and at each of 4 steady levels of exercise (77, 308, 616 and 924 kpm/min), while they breathed 30 % O (in order to minimise chemoreceptor activity) During exercise P rose to 150—180 mm Hg and I fell to 500—350 msec (HR 120—160/min)

In all subjects reflex sensitivity decreased progressively with increasing exercise and was linearly related to the pulse interval immediately prior to injection (Fig 1) The reflex sensitivity, expressed numerically in this way, approaches zero at an exercise tachycardia of 150/min (I = 400 msec) These conclusions do not agree with those of Bevegård and Shepherd (1966) and Robinson *et al* (1966) who in their analysis considered the heart rate response On converting their data to pulse intervals however their results agree with ours The reflex depression we have observed may be partly due to a peripheral interaction between the sympathetic and vagus at the sinus node and partly to central resetting of the arc

References

Bevegård B S and J T Shepherd *J clin Invest* 1966 *45* 132—142

Robinson B F S [illegible] Epstein [illegible] D Beiser and E Braunwald *Circulat Res* 1966 *19* 400—409

Smyth H [illegible] P Sleight and G W Pickering *Circulat Res* 1969 *24* 109—121

C 7

Department of Pharmacology, Karolinska Institutet Stockholm, Sweden

Release of Prostaglandin-like Material from Canine Subcutaneous Adipose Tissue by Nerve Stimulation

By

B B Fredholm, S Rosell and K Strandberg

Prostaglandins have been proposed to play a role as feedback inhibitors of lipolysis evoked by sympathetic nerve stimulation (Shaw and Ramwell 1968) In the present study the venous outflow of prostaglandin like material before, during and after nerve stimulation was determined in canine subcutaneous adipose tissue (Rosell 1966)

An ethanol extract of plasma or adipose tissue was thrice extracted with ether at pH 3 The prostaglandins were then brought over into phosphate buffer at pH 8 The recovery of PGE_1 added to blood was 90 per cent Occasionally further purification by means of column (Samuelsson 1963) and thin layer chromatography (Green and Samuelsson 1964) was carried out The bioassay utilized cross brackets assay on segments of the uterine horn from virgin rats in an 0 5 ml organ bath

The results are shown in Table 1 Prostaglandin like material was not detectable venous blood from unstimulated adipose tissue During and after stimulation activity was found in 7 out of 11 dogs In two experiments the activity was characterized as prostaglandin of the E type The recovery of PGE_1 infused into adipose tissue was found to range between 5 and 17 per cent Prostaglandin content was

Table 1

Dog no	Stimulation freq cps	time min	Prostaglandin release Control	Stim (10 min) ng PGE_1	Poststim (10 min)	Threshold of bioassay ng PGE_1/ml
1	10	30	—	8 2		1
2	10	30	—	—	—	4
(a block)	10	30	—	—	—	4
3	4	8	—	—	7 8	1 5
4	4	5	—	3 1	1 5	0 3
5	10	20	—	—	—	0 5
6	8	21	—	—	—	10
(a block)	[illegible]	21	—	—	—	10
7	6	21	—	—	—	4
8	4	10	—	—	2	0 5
9	4	8	—	—	1 6	0 5
10	4	8	—	—	1 6	0 5
11	5	20	—	—	—	0 5
(a block)	5	20	—	2 6	0 9	0 5

similar in specimens from stimulated and unstimulated adipose tissue (1 3 to 5 5 ng/g tissue)

The results demonstrate that—at least in some experiments—smooth muscle stimulating material similar to prostaglandin is released from adipose tissue into the bloodstream as a consequence of nerve stimulation The amounts found were generally lower than those necessary to produce a significant inhibition of stimulated lipolysis (Fredholm and Rosell 1968) The results furthermore indicate a rapid inactivation of prostaglandins in the tissue

References

FREDHOLM B B and S ROSELL *Acta physiol scand* 1968 *76* 6A
GREEN, K and B SAMUELSSON, *J Lipid Res* 1964 5 117—120
ROSELL S, *Acta physiol scand* 1966 *67* 343—351
SHAW J E and P W RAMWELL *J biol Chem* 1968 *243* 1498—1503
SAMUELSSON B, *J Am Chem Soc* 1963 *85* 1878—1879

C 8

From the Department of Physiology, Karolinska Institutet Stockholm Sweden

Inhibition by Prostaglandin E_1 of the Sympathetic Neuromuscular Transmission in the Rabbit Heart

By

P HEDQVIST and A WENNMALM

Prostaglandin E_1 (PGE_1) has recently been shown to interfere with the sympathetic neuromuscular transmission in the cat spleen and in the guinea pig vas deferens (Hedqvist and Brundin 1969 Euler and Hedqvist 1969) The present study was designed to investigate if a similar effect of PGE_1 could be established in the rabbit heart

Rabbit hearts with intact sympathetic nerve supply were perfused by the Langendorff technique with Tyrode solution The nerves were stimulated supramaximally at a frequency of 10/sec for 30 sec periods at intervals of 15 min The perfusate was collected during the stimulation and the following 90 sec After adsorption on alumina the perfusate was analyzed fluorimetrically for noradrenaline (NA) PGE_1 was infused into the heart to produce final concentrations ranging from 8×10^{-8} to 3×10^{-7} M

In 6 out of 9 expts infusion of PGE_1 caused a marked increase in the perfusion flow rate confirming the observations of Mantegazza (1965) PGE_1 was also found occasionally to produce a positive chronotropic and inotropic response

Infusion of PGE_1 consistently and markedly reduced the outflow of NA in response to nerve stimulation and counteracted the positive chronotropic and inotropic actions of this type of stimulation After cessation of the infusion of PGE_1 all three effects of nerve stimulation returned towards the preinfusion levels

The results reported in this communication suggest that PGE_1 in low concentrations inhibit the transmitter release from the sympathetic nerve terminals of the rabbit heart, and support the concept that PG s of the E series may act as modulators of sympathetic nerve transmission (Hedqvist 1970)

References

EULER U S v and P HEDQVIST *Acta physiol scand* 1969 77 510—512
HEDQVIST, P, *Life Sci* 1970 9 269—278
HEDQVIST, P and J BRUNDIN, *Life Sci* 1969 8 389—395
MANTEGAZZA, P, *Atti Accad Med Lomb* 1965 20 66—72

C 9

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Energy Turnover in Contracting Human Muscle

By

S AKRE and K AUKLAND

The energy expended during muscular contraction is converted to heat and external work During isometric contraction no external work is performed and the whole energy turnover therefore ends up as heat When heat loss from the muscle is prevented, the accumulation rate of heat in the tissue directly reflects the metabolic rate The rate of heat accumulation Q (cal/min g), can be calculated from the rate of temperature rise $Q = \Delta T/\Delta t \cdot s$, where $\Delta T/\Delta t$ is the rate of temperature rise (°C/min) and s is thermal capacity of muscle (≈ 0.9 cal/°C g)

The energy cost of isometric contractions in biceps and triceps muscles has been studied in conscious subjects The force generated by the muscle was measured as deflection of a rigid steel rod Heat accumulation rate during contraction was measured with a small thermocouple sewn into the muscle belly To prevent heat loss by blood flow, circulation was arrested by inflation of a cuff placed proximally on the arm, and heat dissipation to the surroundings was reduced by insulating covers The isometric contractions studied were 1) maintained contractions at a constant force or 2) rhythmic contractions at constant mean force The force was varied over a wide range and related to individual maximal force During both types of contraction the muscle temperature increased linearly, indicating constant heat generation

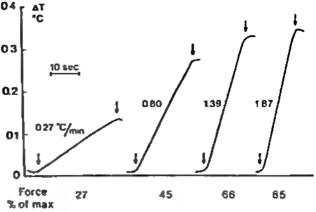

Fig. 1. Temperature rise in triceps during maintained contractions. Generated force calculated in per cent of maximum. Start and stop of contractions marked by arrows.

rate (Fig. 1). The heat accumulation rate was proportional to the generated force, and maximum values for maintained contractions ranged from 1.35 to 1.80 cal/min · g (mean 1.56). For a given mean force, the heat generation was higher during rhythmic than during maintained contractions, and increased with raised contraction frequency. The maximum heat generation during rhythmic contractions exceeded the heat generation in maintained contractions.

C 10

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Tension in Isolated Frog Muscle Fibres during Small Changes in Length

By

J. Lännergren

Results from recent experiments on whole sartorius muscles (Hill 1968) have shown that at rest a special kind of elasticity exists which is due to a component lying between the two sets of filaments. The elastic effect is seen only for very small length changes, up to about 0.2 % of the muscle length, and then an 'elastic limit' is reached. If the length change then continues at a constant velocity, the tension developed is maintained at a fixed level, producing a sort of frictional resistance.

The present experiments were performed in order to see whether similar elastic and frictional effects could be obtained also in isolated fibres. The use of single fibre preparations would also allow more accurate determinations to be made of th

length change necessary to reach the elastic limit Furthermore, the membrane potential can more conveniently be controlled in a single fibre and its effect on the tension response determined.

Single twitch fibres were subjected to slowly applied stretches or releases from an initial sarcomere length of 2 2 μm Length tension relationships similar to those shown by Hill were obtained The frictional force was about 0 4 dyn The elastic limit was reached at 0 18 % length change from L_0 which corresponds to 20 Å/half sarcomere $\pm$ 2 5 (mean of 10 exp/s $\pm$ S D) When alternating stretches and releases were performed the elastic limit was reached at the same length change from the initial position in both directions

According to Hill s working hypothesis a small number of stable cross-bridges exist between actin and myosin filaments at rest The elastic behaviour is due to the spring like properties of these bridges At the elastic limit these bridges give or slip" If this interpretation is correct the individual cross-bridge would have a working range of about 40 Å and is at rest oriented in a midway position

When fibres were depolarized by increasing the K-conc in the surrounding fluid the tension response to a length change was much reduced and no distinct elastic limit could be seen Similar effects were produced by the application of caffeine (1 mM) These results suggest that the stability of the bridges is decreased by procedures known to affect the intracellular Ca-concentration

References

Hill D K *J Physiol* Lond 1968 *199* 637—681

C 11

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Cationic Dependence of Salivary Secretory Potentials

By

O H Petersen

When a salivary gland is stimulated to secrete the basal acinar cell membranes hyperpolarize (secretory potential Lundberg 1955) As it has been shown that the size of the secretory potential is unaffected by the presence of dinitrophenol in a concentration sufficient to inhibit salivary secretion and active potassium uptake (Petersen 1970a) it is tempting to assume that an outward passive transport of cations is responsible for the increase in membrane potential observed during stimulation of the gland.

Cat submandibular glands were perfused with normal sodium and low sodium

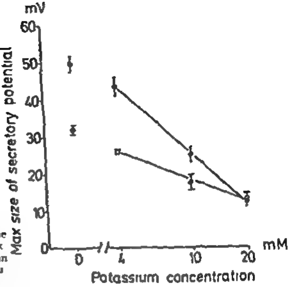

Fig 1 The effect on the size of the secretory potentials of varying the external potassium concentration in sodium ■ and TEA ● Locke perfusion solutions

tetraethylammonium (TEA) Locke solutions with varying potassium concentrations The dependence of the size of the acetylcholine induced secretory potential on the external potassium concentration in the sodium and TEA Locke solutions respectively is shown in Fig 1 In both media the size of the secretory potential decreased with increasing external potassium concentration and increased when potassium was omitted from the perfusion fluid At normal and low external potassium concentrations the sizes of the secretory potentials recorded in TEA solution were markedly greater than the sizes of those recorded in sodium Locke solution

These findings seem to indicate that the secretory potential is predominantly due to an enhanced permeability of the basal acinar cell membrane to potassium allowing this ion to diffuse out of the acinar cells The results with the TEA solutions indicate that during perfusion with sodium Locke solution there must exist a passive inward sodium current partly short circuiting the outward potassium current. This hypothesis fits well with the recent finding (Petersen 1970b) that the stimulation induced potassium loss from the cat submandibular gland is severely reduced during perfusion with TEA Locke solution

References

Lundberg A *Acta physiol scand* 1955 *35* 1—25
Petersen O H *Acta physiol scand* 1970a. In press
Petersen, O H *J Physiol* (Lond.) 1970b I

C 12

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Inhibition of Peripheral Utilization of Thyroid Hormones Induced by Lithium

By

G OHLIN and U SODERBERG

This investigation was prompted by the well known observation that lithium treatment of psychiatric illness induces goitre (Schou *et al* 1968) According to Sedvall *et al* (1968) lithium reduces the protein bound iodine and increases thyroid uptake of radioactive iodine Since lithium also influences electrolyte and water distribution (Murphy and Goodwin 1969), it seemed reasonable to investigate whether or not lithium goitre is a sign of iodine shortage caused by increased renal iodide loss

Food and water consumption salt appetite and weight development were studied in rats as basic indices of thyroid hormone activity One group of animals were thyroidectomized to permit measurement of the biological half life of ^{131}I labelled thyroxine and iodide that was done with a whole body counter Thyroidectomy was performed to abolish thyroid uptake of iodide After operation the animals were divided into two groups one receiving lithium with a stomach tube Dihydrochysterol replaced loss of parathyroid activity

Short term treatment with lithium increased the renal iodide loss significantly prolonged treatment seemed to give the opposite effect because the iodide loss was more augmented in the controls than in the lithium animals This difference could be ascribed to more rapid development of thyroid hormone deficiency in the thyroidectomized controls with increased renal loss of chloride and iodide and development of salt appetite as a consequence *(cf* Fregly and Taylor 1964) In favour of the hypothesis that lithium prolonged the survival of endogenous thyroid hormones the lithium animals also had higher food consumption than the controls either when offered free food and water or only food during water deprivation On free diet lithium-treated animal gained in weight the controls showed insignificant weight loss Similar differences in appetite and thirst were seen also when euthyroid and thyroidectomized rats were compared The biological half life of ^{131}I labelled thyroxine was also markedly prolonged by lithium and after repeated thyroxine administration the radioactivity cumulated to higher levels in the lithium animals than in the controls

Even if increased iodide loss occurred in the early phase of lithium treatment it seems difficult to evaluate whether or not this result is a support of the assumption that lithium goiter is caused by iodine shortage The finding of markedly prolonged biological half life of thyroxine when measuring elimination of labelled hormone as well as food water and salt consumption shows that a considerable loss of iodine will

be outbalanced by reduced need of hormone synthesis If the pituitary serves to maintain constant blood level of thyroid hormones by means of negative feed back control, a decreased peripheral utilization of hormone, as after lithium, would lead to compensatory reduction of thyroid activity through diminished TSH output Recently, Cooper and Simpson (1969) showed that lithium lowers thyroid secretion to a level below that expected merely from reduced hormone utilization blood levels of both free and protein bound hormone being decreased

References

Cooper, T B and G M Simpson *Curr Therap Res* 1969 *11* 603—608

Fregly, M J and R E Taylor, Jr, In *Thirst* Ed *M J Wayner*, Pergamon Press, Oxford 1964, pp 139—172

Murphy, D L F K Goodwin and W E Bunney, Jr., *Lancet* 1969 2 458—461

Schou, M, A Amdisen, S E Jensen and T Olsen, *Brit Med J* 1968 *3* 710—715

Sedvall, G, B Jonsson, U Pettersson and K Levin, *Life Sci* 1968 7 1257—1259

C 13

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Plasma Progesterone Levels in Goats during Pregnancy Measured by the CBG-(Corticosteroid-Binding Globulin) Method

By

A-K Blom and O Lyngset

Plasma progesterone in goats was measured by the CBG-method described by Neill *et al* (1967) The progesterone concentration in peripheral vein blood and ovarian vein blood from 16 goats killed at various stages of pregnancy showed an increase in the early stage of pregnancy Peak values were reached at about 90 days with a subsequent decline Just prior to parturition low values were obtained For peripheral blood maxima and minima of 33 and 3 ng/ml plasma were recorded on the 26th and 145th day of pregnancy Blood from the ovaries gave values between 3100 ng/ml plasma on the 90th day and 595 ng/ml plasma on the 140th day The decline in peripheral plasma progesterone was found statistically significant ($p < 0.001$ $t = 5.36$) when averages obtained for the time intervals 60—105 days (27.1 ± 2.3 ng/ml)and 120—145 days (12.1 ± 1.6 ng/ml) were compared

Peripheral plasma progesterone decreased significantly 10 *minutes after* extirpation of the uterus and the ovaries Average decrease 6.8 ± 2.0 ng/ml ($p < 0.01$) In 1968 Linzell and Heap claimed that for two goats examined, the uterus removed

significant amounts of progesterone during pregnancy These results are confirmed by us for 6 goats in late pregnancy The uterine progesterone consumption seemed to decrease near term In two goats sampled before and after parturition the plasma progesterone concentration was found to decrease prior to parturition and remained low for at least three days after parturition

It is concluded that in pregnant goats the ovaries are the main source of progesterone production and that the uterus or its contents consumes progesterone during pregnancy

References

NEILL J D E D B JOHANSSON J L DATTA and E KNOBIL *J Clin Endocr* 1967 *27* 1167—1173

LINZELL J L and R B HEAP *J Endocr* 1968 *41* 433—438

C 14

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Sodium-Angiotensin Synergism in Central Regulation of Water and Salt Metabolism

By

B ANDERSSON and O WESTBYE

Infusions of hypertonic NaCl into the 3rd ventricle of goats may elicit drinking release of antidiuretic hormone (ADH) and natriuresis (*cf* Andersson Dallman and Olsson 1968) In the rat thirst is also induced by intravenous and intrahypothalamic application of angiontensin (*cf* Fitzsimons and Simons 1969) Therefore it appeared of interest to study how intraventricular infusions of angiontensin alone or together with hypertonic NaCl would affect water and salt metabolism

Slow (10 μl/min) infusions into the 3rd ventricle were made in not hydrated and hydrated goats Angiotensin (Hypertensin Ciba) was solved in 0 12 M or in 0 33 M NaCl and was infused at 1 5—2 ng/kg min For comparison infusions of 0 33 M NaCl without angiotensin were also made

In *not hydrated goats* 30 min infusions of 0 33 M NaCl or of angiotensin in hypotonic saline caused cumulative drinking of 1 to 2 l of water and a weak natriuretic response In contrast the infusion of angiotensin together with hypertonic NaCl induced drinking of 4 to 6 l and extreme natriuresis

In *hydrated goats* 30 min infusions of 0 33 M NaCl or of angiotensin in hypotonic saline caused practically no drinking and only moderate elevation of renal Na excretion However also here the combination of angiotensin and hypertonic NaCl

acted as a very powerful stimulus to thirst and natriuresis Brief (5 min) infusions of 0 33 M NaCl, or of angiotensin in hypotonic saline caused no inhibition of the water diuresis However, 5 min infusions of angiotensin in 0 33 M NaCl inhibited the water diuresis for 40 to 60 min During this antidiuresis urine osmolality rose considerably, implying release of ADH

It has been suggested that hypothalamic "osmoreceptors" in Verney's sense (1947) regulates both ADH release and thirst However, infusions of hypertonic glucose or saccharose into the 3rd ventricle do not seem to elicit thirst or ADH release (Andersson *et al* 1968, Olsson 1969) An elevated Na^+ concentration may therefore stimulate these mechanisms more efficiently than extracellular hyperosmolality as such The present experiments show that angiotensin mimics and potentiates all effects of hypertonic NaCl infused into the 3rd ventricle It might be that angiotensin changes (facilitates?) Na^+ transport into neurons regulating water and salt metabolism If so, intracellular, rather than extracellular Na^+ concentration may determine the activity of these neurons

References

Andersson B M F Dallman and K Olsson *Proc Int Congr Physiol, Wash* 1968 vol *VI* 200—201
Fitzsimons J T and B J Simons *J Physiol* (Lond) 1969 *203* 45—57
Olsson K., *Acta physiol scand* 1969 *77* 465—474
Verney, E B, *Proc Roy Soc B*, 1947 *135* 25—106

C 15

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The Effect of Gamma Fibre Block on Afferent Muscle Nerve Activity during Voluntary Contractions

By

K E Hagbarth, A Hongell and B G Wallin

Afferent muscle nerve activity in the median nerve was recorded on human volunteers by the method described by Hagbarth and Vallbo (1968) The tungsten microelectrode was placed in muscle nerve fasciculi from the pronator teres or the finger flexor muscles proximal to the elbow Both single and multi unit recordings were obtained and always the nerve response to local pressure against the muscle passive stretch vibration and voluntary contraction was tested From such tests sometimes complemented with electrically induced muscle twitches most single units could be identified as muscle spindle primary afferents (Ia fibres) The response pattern of the multi unit recordings was similar and consequently it was concluded that multi unit recordings were dominated by Ia afferents This report is largely based on results from multi unit recordings

The typical response to suddenly applied sustained local pressure or passive muscle stretch was of a predominantly phasic type with an initial pronounced burst of impulses succeeded by a weak, inconstant static discharge During rapid alternating "isotonic" contractions a double peak response was obtained with one burst of activity during the contraction and one during the stretch phase, whereas "isometric" contractions resulted in a continuous high level of activity during the whole contraction The same tests were repeated after having injected 15—30 cc dilute lidocaine solution (0 5 % Xylocain, Astra) proximal to the electrode, supposedly anaesthetizing only thin nerve fibres including the gamma efferents (Matthews and Rushworth 1957)

After the anesthesia no alteration of the nerve impulse pattern was seen during passive stretch or local pressure However, the response to voluntary contractions was markedly changed Instead of the typical double peak response during rapid alternating isotonic movements, only the stretch response remained, the contraction burst being greatly diminished Also, the sustained afferent nerve response normally accompanying isometric contractions was very much reduced after the lidocaine block, indicating a lack of fusimotor activation of the muscle spindles during the contraction.

The nerve block also resulted in a reduction of maximal muscle strength and muscle vibration tests revealed that this reduction of maximal voluntary power at least in part depended upon a lack of autogenetic excitatory inflow from the spindles The power could namely to a relatively large extent be restored by stimulating the muscle spindles artificially with high frequency mechanical muscle vibration (Hagbarth and Eklund 1966)

From the results it is concluded that there is little or no fusimotor activity influencing the muscle spindles in the voluntarily relaxed muscle However, in the voluntarily active muscle gamma nerve activity is of considerable importance for normal motor performance

References

Hagbarth K E and G Eklund In *proceedings of the first Nobel Symposium* (Ed R Granit) Almqvist and Wiksell Stockholm. 1966 177—186

Hagbarth K E and A B Vallbo *Exp Neurol* 1968 22 674—694

Matthews, P B C and G Rushworth *J Physiol* (Lond) 1957 *135* 245—262

C 16

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Reproducibility of Electromyographic Measurements with Inserted Wire Electrodes and Surface Electrodes

By

E R Buskirk and P V Komi

Despite careful standardization of testing conditions quantitative differences has been observed in repeated electromyographic (EMG) measurements (Jonsson and Reichmann 1968) In order to obtain statistical information about the differences a study was conducted with inserted wire electrodes and surface electrodes in experimental conditions where both submaximal and maximal isometric and isotonic contractions of m biceps brachii were used

In the first part of the experiment 8 male college students were tested isometrically on three different testing days with one day of rest between each Integrated EMG readings were taken twice on each day at the following isometric tensions 20, 40 60, 80, and 100 % of maximum The recordings made with surface electrodes (11 mm in diameter, E & M Instruments Co) showed good reliability (r= 85—95) at all levels of tension, if the comparisons were made between the tests of the same day The reliability coefficients were somewhat lower, yet satisfactory, in day to-day comparisons The wire electrodes, which were prepared and inserted according to the method of Basmajian and Stecko (1962) showed satisfactory reproducibility of measurements during the same day However, when reinsertion was required (day-to-day comparison) the reproducibility of recordings was poor

Further experiments were made with surface electrodes in isotonic conditions (n= 29) A special electrical dynamometer (Komi *et al* to be published) was used to keep the velocity of muscular contraction (m. biceps brachii) constant both in con centric and eccentric conditions At the velocity of 25 mm/sec the day to-day com parison during maximal contractions showed high reliability of EMG recordings (r> 90)

It is concluded that the surface electrodes, but not the wire electrode technique used can be utilized reliably in experiments where EMG recordings are repeated at intervals of several days

References

Basmajian J V and G Stecko, *J Appl Physiol* 1962 17 849
Jonsson B and E Reichmann *Acta Morph Neederl Scand* 1968 7 73—90

C 17

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Electron and Light Microscopic Evidence for a Light-Induced Pupil Reaction in the Apposition Eye of the Blowfly

By

G Gemne and G. Seitz

Light- and dark-adapted eyes of the normal blowfly, *Calliphora erythrocephala* (Meig), were investigated Preliminary results — obtained from freeze-etching replicas — show that, in the sense cells no 1 to 6 of the dark-adapted eye, an approximately 1 μ thick layer of vesicles borders on the rhabdomeres These vesicles — which are very few in the light-adapted eye — seem to arise by pinocytosis at the bases of the rhabdomeric microvilli, thus assimilating part of the low-refractive medium (Seitz 1968) of the axial retinular space Using the Becke technique on formaldehyde-fixed, unstained, 20 μ thick cross-sections of the eye, it could be observed light microscopically that, in the dark-adapted retinular cell, the vesicles decrease the optical density of the cytoplasmic medium adjacent to the rhabdomere The light-adapted sense cells were, however, seen to be optically homogeneous In the retinular cells nos 7 and 8, and in the axial retinular space, no morphological or optical differences could be observed between the two states of adaptation

Because the tips of the rhabdomeric prolongations lie in the focal plane of the dioptric system (Seitz 1968), the ratio between the optical densities of the rhabdomere and the adjacent cell medium defines an area in the principal plane on the object side Only the paraxial rays passing through this area will be transmitted in the rhabdomere, which acts as a wave guide Calculations based on geometrical optics show that, in comparison with the light-adapted state, rhabdomeres no 1 to 6 of the dark-adapted eye will transmit about 1 3—1 7 times more light energy (Seitz, to be published) Therefore the present results show the existence of a light-induced pupil reaction correlated to ultrastructural changes in the photoreceptor cells of the apposition eye

References

Seitz, G, Z vergl Physiol 1968 59 205—231

C 18

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Release of Vasopressin from a Rat Supraoptico-Neurohypophysial System in vitro

By

P Eggena and N A Thorn

Isolated systems containing the neural lobe, the neural stalk, the supra-optic nuclei, and smaller or larger parts of adjacent areas (but lacking the adenohypophysis) were incubated in a McIlwain Rodnight buffer The rate of release of vasopressin into the incubation medium was determined by an antidiuretic assay method with i v hydration Preparations including the adjacent areas released less vasopressin in the equilibration period following the isolation procedure than did preparations without these structures The baseline rates of release reached by the systems were much smaller than those of isolated neural lobes Acetylcholine (1 mg/l) and strongly hypertonic sodium chloride media both enhanced the rate of release of vasopressin from about 20 to more than 100 pg/min Cross tachyphylaxis was noted with these two forms of stimulation after less than 1 % of the total vasopressin content of the system had been released A concentration of potassium in the medium of 56 mmoles/l induced release of much larger amounts of vasopressin than did the other stimuli

C 19

Department of Pharmacology Karolinska Institutet, Stockholm Sweden

Quantitative Correlation between Degranulation and Histamine Release Following Exposure of Rat Mast Cells to Compound 48/80 in vitro

By

R Nosal, S A Slorach and B Uvnäs

The quantitative relationship between the release of histamine, ^{35}S labelled heparin and granule protein from rat mast cells exposed to compound 48/80 *in vitro* has been investigated in order to elucidate the mechanism of action of this releaser

The cells were taken from rats injected 2 3, 6, 12 or 20 days earlier with $Na_2{}^{35}SO_4$ It was demonstrated by chromatography on Dowex 1-X2 that the ra

activity (^{35}S) in mast cells taken after these intervals was almost exclusively present in heparin molecules Since it is known that virtually all the heparin in mast cells is located in the granules a study of the ^{35}S release will provide valid information about the release of granules provided that the ^{35}S labelled heparin is evenly distributed with respect to the granules

A good correlation was found between the three release curves From studies of the ^{35}S/protein ratio in the extruded granules and granules obtained by water lysis of the washed compound 48/80 treated cells it appears that the ^{35}S labelled heparin was first evenly distributed with respect to the granule protein 12 to 20 days after injection Using cells taken after such an interval the ratio percentage histamine release/percentage ^{35}S release was found to be 1 1—1 4

We conclude from these results and from the similarity between the time courses of histamine and ^{35}S release that the mechanism of histamine release induced by compound 48/80 involves an initial extrusion of histamine containing granules followed by an exchange of histamine in the extruded granules and cations in the extracellular medium

C 20

Institute of Physiology University of Lund Sweden

Origin and Termination of the Cuneocerebellar Tract in the Cat

By

J D Cooke B Larson O Oscarsson and B Sjolund

The cuneocerebellar tract consists of one component excited by cutaneous and high threshold muscle afferents (cutaneous component) and one component excited by group I muscle afferents (Holmqvist Oscarsson and Rosen 1963) Activation of the cutaneous component evokes intense activity in neurones of the cerebellar cortex whereas activation of the group I component only evokes weak activity (Korlin and Larson 1970) This observation supplies the first evidence suggesting that different mossy fibre paths may have different modes of termination The present experiments were performed in order to ascertain that the two components terminate in the same cortical area and to identify the cells of origin Previous observations indicated that the group I component but not the cutaneous component originates from cells in the external cuneate nucleus (Rosen 1969)

Cuneocerebellar neurones were identified by their antidromic activation on stimulation of the surface of the cerebellar anterior lobe (Lundberg and Oscarsson 1960) and by their orthodromic activation on stimulation of muscle and skin nerves in the

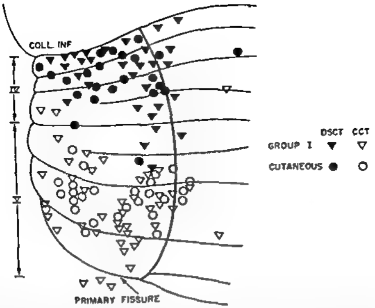

Fig 1 Termination of group I and cutaneous components of the cuneocerebellar tract (CCT) as indicated by low threshold foci for antidromic activation Corresponding data for the dorsal spinocerebellar tract (DSCT) are also shown (Lundberg and Oscarsson 1960)

ipsilateral forelimb It was demonstrated that neurones belonging to both components terminate intermingled in the same cortical area (Fig 1) and have thresholds and latencies in the same range on antidromic stimulation The cell bodies of the group I component were found in the external cuneate nucleus (Rosen 1969) and those of the cutaneous component more ventrally and medially in a rostral portion of the main cuneate nucleus It can be concluded that the two components terminate in the same cortical area but carry different kinds of information and have different origins and modes of termination

References

HOLMQVIST B O OSCARSSON and I ROSEN *Acta physiol scand* 1963 *58* 216—235

C 21

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Rhythmic Thalamic Unit Activity in the Unanesthetized Cat

By

L I Gjerstad and K K Skrede

During barbiturate anesthesia thalamic nuclei serve as pacemakers for cortical spindle activity (for a review see Andersen and Andersson 1968) Certain stages of natural sleep are characterized by a cortical activity which is similar to the barbiturate spindles By analogy, it can be postulated that these sleep stages are associated with rhythmic activity of the thalamus as well We have tested this hypothesis by recording unitary activity from thalamic neurones in unanesthetized cats

A PVC cylinder was firmly fastened to the skull with screws and dental acrylic, over a trephine hole (diameter 20 mm) This cylinder was used to fix the animal to a frame in which it was trained to stay quiet (Fig 1 A) The training required 1—3 weeks Tungsten microelectrodes could penetrate the intact dura anywhere inside the cylinder by rotation of the two covers closing the cylinder (Evarts 1968)

Spindle like thalamic activity could be recorded from the unanesthetized cat preferentially during drowsiness and light sleep Rhythmic bursts of unit discharges occurred at a frequency of 5—14/sec The frequency was highest during light sleep During these stages the corticogram showed spindle like activity of a frequency similar to that of the rhythmic thalamic activity Fig 1B shows a multiple unit recording from the VPL nucleus obtained when the cat appeared drowsy The bursts of spikes are superimposed on negative waves which are followed by positive deflections This activity is similar to the activity recorded from the same group of cells 30 min after the injection of 30 mg/kg of sodium pentobarbital (Fig 1 C)

During conditions of alertness most thalamic cells had a higher firing rate than during drowsiness and sleep and no spindle like activity was seen During REM sleep, the thalamic activity was similar to the pattern observed during alertness and the neuronal firing rate was even higher

References

Andersen P and S A Andersson *Physiological basis of the alpha rhythm* Appleton Century Crofts New York Neuroscience Series No 1 1968 235 pp

Evarts E V *Electroenceph clin Neurophysiol* 1968 24 83—86

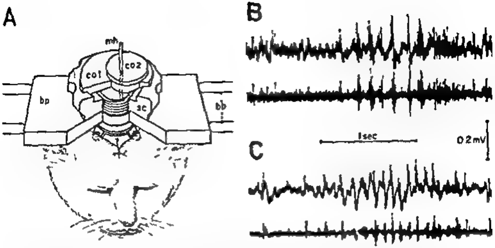

Fig 1 *A* The cylinder (hatched) is attached to a brass plate (bp) by a threaded screw (sc) Four brass bars (bb) connect the plate to the animal frame The microelectrode holder (mh) slides through a hole in the inner of two perspex covers (co1 and co2) *B* Multiple unit recording, showing spontaneous and rhythmic … unanesthetized, drowsy cat In the uppe… of 200 msec to record the slow waves … a time constant of 2 msec *C* Activity … administration of 30 mg/kg sodium pentobarbital intraperitoneally

C 22

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Some Aspects of Proprioception after Forebrain Commissurotomy in Man

By

U Norrsell[1]

Sensations regarding the position or movements of parts of the body, *proprioception* or kinesthesia, depend on a complex coordination of intero- and exteroceptive afferent, as well as efferent activity (Merton 1964) It has been assumed that the cortical projection from joint afferents from which conscious sensations presumably can be evoked selectively, is especially important Proprioception has now been examined in patients whose forebrain commissures had been transected for therapeutic reasons (Bogen, Sperry and Vogel 1970) The most striking symptom after such commissurotomy is *the tendency for each of the two disconnected hemispheres to function* autonomously with respect to higher nervous functions

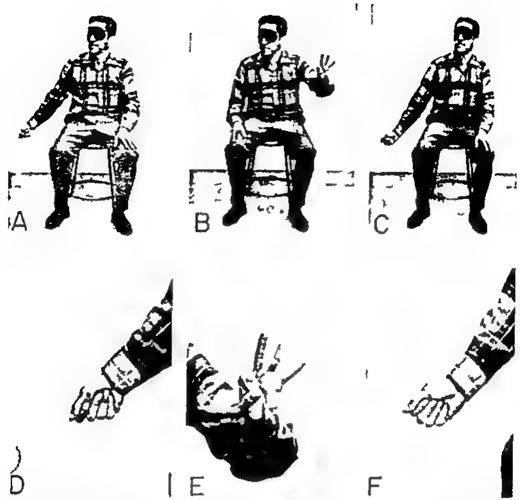

Fig 1 Patient two and a half years after transection of the corpus callosum and the anterior commissure In A the patient is holding the right arm and hand in a position arranged by the experimenter In B the patient claims to be showing the position previously held by the right arm and hand using the left side instead In C the patient has returned to the original position to show that he remembers it D E and F show the handpositions of the pictures above in magnification

A division of the position sense was indeed evident in some of the patients Fig 1 The patient could memorize and return to a given position with the right arm (A and C) but was unable to imitate that position with the other arm (B) This inability (B) was not due to any impairment of the left side since a position held by that arm could be equally well repeated although in that case not by the right side Thus 'position sense as tested above utilizes information available in one hemisphere only ((See also Sperry Gazzaniga and Bogen 1969) At least one patient did not show as marked a division of the position sense presumably because a compensatory development of the ipsilateral motor system made him able to base motor activity of both sides on information available to one hemisphere

However if the patients were required to perform a wilful act involving placing of both arms and hands they failed to show any behavioural dichotomy (*cf* Mark and Sperry 1968) For example, the patient of Fig 1 could be asked when blindfolded to gauge a distance or to locate an object with one hand and having done so to place the second hand on the first Both acts were performed accurately Apparently he was now able to utilize information not available under the circumstances described above Thus position sense in the meaning ' ability to utilize knowledge of position in wilful acts either utilizes information available to both hemispheres or an afferent efferent coordination performed at a subhemispheric level

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References

Bogen J E, R W Sperry and P J Vogel *Basic Mechanisms of the Epilepsies* Ed Jasper H H A A Ward and A Pope Little Brown and Co In press

Mark R F and R W Sperry *Exp Neurol* 1968 *21* 92—104

Merton P A *Symp Soc exp Biol* 1964 *18* 387—400

Sperry R W M S Gazzaniga and J E Bogen *Handbook of Clinical Neurology* Ed Vinken P J and G W Bruyn North Holland Publishing Co 1969 *4* 273—290

C 23

Department of Physiology Gymnastik och Idrottshoegskolan Stockholm Sweden

Lactate Accumulation in the Working Muscles of Man

By

J Karlsson and B Saltin

High values for blood lactate concentration have been obtained after short time (2—4 min) maximal work loads and have empirically been found to correlate closely with exhaustion However as suggested by Asmussen *et al* (1948) maximal performance may be limited not by critical values of lactate concentration in the blood but in the working muscles

Actual data on local lactate concentration in the deep part of vastus lateralis of M quadriceps femoris on man may support this suggestion (Karlsson *et al* to be published) To further study the dynamics of the lactate accumulation in the working muscle groups 3 subjects performed maximal exercise of 2 ■ and 17 min duration on an electrically braked ergometer In further experiments the two longer maximal work periods were interrupted after 2 min and after 2 and 6 min respectively Biopsy specimens were obtained according to Bergstrom (1962) immediately after the work load was terminated for determination of ATP CP glycogen and lactate (Lowry *et al* 1964) Blood samples for blood lactate determination (Scl

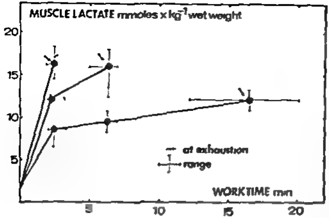

Fig 1 Mean lactate concentration in the lateral portion of M quadriceps femoris for three subjects during and immediately after heavy exercise leading to exhaustion after 2 6 and 17 min The values after 2 and 6 min in the longer runs were obtained in separate experiments where the exercise was stopped at these points

1959) were drawn from a prewarmed fingertip simultaneously Further samples were collected to secure peak blood lactate concentration (Åstrand 1960)

The results showed that the break down of the phosphagens (ATP and CP) were maximal whether the subjects after 2 and 6 min work were exhausted or not The accumulation of lactate in the muscle as well as in the blood however as illustrated in Fig 1 continuously increased until exhaustion After 2 and 6 min maximal exercise higher lactate concentrations were obtained than after 17 min in the muscle as well in the blood

It is therefore concluded that if the muscle tissue lactate concentrations were the n for exhaustion after 2 and 6 min respectively some additional factor must be sent at exhaustion after 17 min when maximal lactate values neither in the muscle nor in the blood were attained

References

Asmussen E W v Döbeln and M Nielsen *Acta physiol scand* 1948 *15* 57—62
Åstrand I *Acta physiol scand* 1960 *49* Suppl 169 1—92
Bergström J *Scand J Clin Lab Invest* 1962 *14* Suppl 68 1—110
Lowry O H J V Passonneau F X Hasselberger and D W Schulz *J Biol Chem* 1964 *239* 1 18—30
Scholtz R H Schmidtz T Bucher and J O Lampen *Biochem Z* 1959 *331* 71—86

C 24

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Free Fatty Acid Mobilization During and After Graded Exercise

By

E D R Pruett

In order to study differences in FFA mobilization, both during and after exhausting exercise of different intensities, subjects were exercised at work loads of rest, 20 %, 50 %, 70 % and 85—90 % of their maximal oxygen uptake (max V_{O_2}). Antecubital blood was drawn before, during and after exercise and after an intravenous glucose infusion which was administered 15 min after the conclusion of exercise. During exercise of up to 70 % max V_{O_2}, plasma FFA increased during exercise. Blood lactate concentrations increased very little during these work loads. During work at 85—90 % max V_{O_2} plasma FFA levels fell during the first 10 min and remained low as long as work continued. Blood lactate concentrations increased to about 80 mg/100 ml. After work stop, plasma FFA showed a dramatic increase. An infusion of 300 mg glucose/kg body weight, which caused an immediate rise in blood glucose and plasma immunoreactive insulin (IRI) concentrations, induced a simultaneous reduction in plasma FFA levels after all exercise work loads. As soon as blood glucose and IRI levels had returned to the preexercise, plasma FFA rose again. This rise was directly dependent on the severity of the preceding work load. After 85—90 % max V_{O_2} (Fig 1), this rise was to a level as high or higher than that immediately before in-

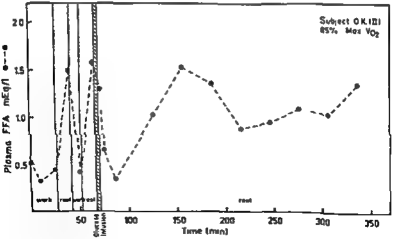

Fig 1 Plasma FFA levels before, during and after very severe bicycle ergometer exercise, and for 4 1/2 hrs following an infusion of 300 mg glucose/kg body weight

fusion FFA levels then remained elevated during at least the next 4 1/2 hrs It was concluded that at least one of the FFA mobilizing factors which are induced by severe muscular exercise is extremely long-lived and that its potency is directly dependent upon the intensity of the severity of the exercise work load in relation to the subject's max V_{O_2}

C 25

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Effect of Electrical Stimulation on Glycogen Synthesis in an Isolated Muscle Preparation

By

S Adolfsson

Increased glycogen content has been observed in skeletal muscle of trained animals and humans After exercise glycogen synthesis is markedly enhanced (Bergström *et al* 1967) The mechanism for this effect is still vaguely elucidated It is highly probable, however, that this stimulation of glycogen synthesis is mediated by an increase in activity of the glycogen synthetase enzyme (UDP-glucose α-1, 4-glucan -4 glucosyl-transferase) since this enzyme is known to be rate-limiting for glycogen synthesis during many conditions The enzyme exists in two forms, one which is dependent on glucose 6-P for its activity (D form), and another which is independent of glucose 6-P (I-form) These forms are interconvertible Enzyme activity is increased when D-form is converted to I-form

Danforth (1965) has reported that a D→I conversion takes place in the synthetase enzyme in mouse hindleg muscle when this muscle is stimulated electrically *in situ* He did not however study the rate of glycogen synthesis and since the experiments were performed *in vivo* the influence of other factors such as epinephrine and insulin could not be excluded This latter difficulty is ruled out in an *in vitro* system We have now started experiments to study the effect of electrically induced contractions on glycogen synthesis and synthetase enzyme in an isolated muscle preparation

The levator ani muscle from prepubertal male rats has been used The muscles were isolated with intact fibres incubated and stimulated electrically according to Arvill (1967) Total muscle glycogen content incorporation of glucose-C^{14} into glycogen and synthetase enzyme activities were analyzed Stimulation was given for 10 minutes with a frequency of 100 impulses per second

Immediately after the period of contraction the muscle glycogen was reduced to one third of that of the controls and the rate of incorporation of glucose C^{14} (CPM per mg tissue) was increased more than three times The percentage of the enzyme

present as I form was doubled Also after the period of contraction glycogen synthesis was enhanced and was observed to be significantly higher even at four hours afterwards At that time the I form however was not increased compared to controls and the glycogen contents of the stimulated muscles did not exceed those of the controls

Thus the stimulatory effect of electrically induced muscular contractions on glycogen synthesis was demonstrated *in vitro* Only initially this stimulation seems to be mediated by an activation by means of a D→I conversion in the synthetase enzyme system

References

ARVILL A *Acta endocr* (Kbh) 1967 56 Suppl 122 27—41
BERGSTROM J L HERMANSEN E HULTMAN and B SALTIN *Acta phys ol scand* 1967 *71* 140—150
DANFORTH W *J B ol Chem* 1965 *240* 588—593

C 26

From The Inst tute of Phys ology Odense University Denmark

Autoregulatory Response in Canine Subcutaneous Adipose Tissue

By

P ALSTRUP NIELSEN and N J SECHER

The purpose of the present study was to investigate the extent of the autoregulation in the adipose tissue as earlier experiments (Nielsen and Secher 1970) might indicate only a minor autoregulatory response

The inguinal subcutaneous adipose tissue in pentobarbital N O/O anesthetized and heparinized dogs was isolated with intact blood and nerve supply (Oro Rosell Wallenberg 1965) The venous effluent was measured by a drop recorder unit and the blood returned to the animal by a catheter in the femoral vein The temperature was kept constant and the blood pressure measured continuously Four types of experiments were performed

After placing a tourniquet around the abdominal aorta the pressure flow values were measured during stepwise hypotension before and after denervation A direct relationship betwen pressure and resistance was found which demonstrates an autoregulatory mechanism

After occlusion of the artery to the fat pad there was a greater hyperemia after 3 min than after 1 min averaging 150 and 180 per cent of control flow respectively ($p<0.05$) There was no significant difference between the hyperemic

responses before and after denervation The greater hyperemia during longer lasting occlusion is in accordance with a metabolic type of autoregulation

By elevating the venous pressure 10 mm Hg, resistance at equilibrium was increased about 40 per cent without falling tendency during 2—4 min which should have given evidence for accumulating vasodilatator agents The vascular response to venous outflow pressure elevation could be of myogenic nature There seems to be a possibility for both myogenic and metabolic type of autoregulation in the adipose tissue If the postocclusive hyperemic blood flow for several different tissues is taken as evidence for the degree of autoregulation (Green and Kepchar 1959), the adipose tissue is not as reactive as the muscular tissue but reacts more than the mesenteric vascular bed

A stepwise haemorrhage was performed by bleeding from a femoral artery to a reservoir The calculated resistance at each pressure step compaired with the values obtained by local hypotension showed an increase in resistance down to 60 mm Hg below which the autoregulatory mechanism seemed to act very forcefully and was able to lower the resistance

References

ALSTRUP NIELSEN P and N J SECHER *Life Sci* 1970 *9* Part I 75—82
GREEN H D and J H KEPCHAR *Physiol Rev* 1959 *39* 617—686
ORO L S ROSELL and L R WALLENBERG *Nature* 1965 *205* 178

C 27

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Background of Increased Flow Resistance and Vascular "Reactivity" in Spontaneously Hypertensive Rats

By

B FOLKOW M HALLBACK, Y LUNDGREN and L WEISS

In 15 expts the hindquarters of a spontaneously hypertensive rat (SHR Okamoto strain) and a matched normotensive control rat (NCR) were simultaneously perfused at constant flow with oxygenated plasma substitute In agreement with earlier results (Folkow *et al* 1969) concerning the entire systemic circuit, flow resistance in the hindquarters of SHR was raised even at maximal dilatation ($p<0\,001$) and almost in proportion to their raised blood pressure Graded noradrenaline (NA) infusions showed that the NA threshold was identical in both groups but the SHR displayed a steeper curve relating the NA dose to the resistance response ($p<0\,001$) as well as a raised maximal pressor response ($p<0\,001$) The same appeared to be true also for other vasoconstrictor agents

The curves for SHR and NCR, *relating NA dosage to flow resistance* from maximal dilatation to maximal constriction, were compared with mathematically deduced curves characterizing two hypothetical resistance vessels, assumed to be in all respects identical except for a 30 per cent increase of media thickness in one of them, encroaching upon its lumen even at complete relaxation. *The deductions show* that the enlarged muscle thickness of such a hypothetical vessel raises proportionally both the curve steepness and the maximal pressor response, despite an unchanged responsiveness and tension development of individual contractile elements.

This comparison between the experimental results and the model deductions revealed that *all* the basic functional characteristics of the SHR resistance vessels observed may be explained by a wall thickness increase of the mentioned type and order of magnitude. No other type of vascular change, structural or functional, can *alone* produce all the mentioned characteristics. *Such a structural change, while allowing* for an undiminished range of resistance adjustments, may account for the entire rise of resistance during rest as well as for the increased vascular ' reactivity '.

References

Folkow, B., M. Hallbäck, Y. Lundgren and L. Weiss: *Acta physiol. scand.* 1969. Suppl. 330. 94.

C 28

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Increased Excitability of Aortic Baroreceptors during Infusion of Noradrenaline

By

H. Aars

Application of noradrenaline to the carotid sinus is known to increase the activity in thin baroreceptor fibres, but to reduce activity in the thick baroreceptor fibres to a degree corresponding to the constriction of the sinus. The relationship between aortic diameter and integrated activity in the whole aortic nerve (which is dominated by activity in thick fibres) is largely unchanged by i.v. infusion of noradrenaline (1—2 μg/kg min) (Aars 1969). To study this problem more closely, the effects of increased smooth muscle contraction on aortic baroreceptors have been examined in single fibre preparations of the aortic nerve in rabbits.

The animals were anesthetized with chloralose and urethane. Trachea was opened to secure free airways, but respiration was not assisted. The diameter of ascending aorta was measured with ultrasonic technique by means of piezoelectric crystals

implanted one week prior to the experiment Action potentials in single baroreceptor fibres were amplified displayed on the oscilloscope and recorded on a jet ink writer Blood pressure was measured in the right common carotid artery Stepwise changes of arterial blood pressure were produced by altering the pressure in a reservoir connected to the abdominal aorta

In 20 single fibre preparations the discharge frequencies of thick fibres when related to arterial blood pressure were either reduced or unaffected by i v infusion of noradrenaline (4—6 μg/kg min) Aortic diameter, measured in late diastole, simultaneously decreased 3—12 %, and the pulsatile diameter variations also became smaller Even so, activity in single fibres was greater than normal when related to diastolic diameter

The results of this and of a previous investigation (Aars 1969) suggest that the effects of noradrenaline on activity of the thick baroreceptor fibres in the aortic nerve depend on the degree of smooth muscle contraction The excitability of the receptors may be increased if the infusion rate of noradrenaline is sufficiently large

References

AARS H *Acta physiol scand* 1969 *76* 27A

C 29

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Evidence against the Role of the Peripheral Chemoreceptors in the Diving Bradycardia in Ducks

By

B HOLM and S C SORENSEN

Experiments were performed to test the hypothesis that the diving bradycardia in the duck is mediated *via* the peripheral chemoreceptors

The effect of peripheral chemoreceptor stimulation on respiration and heart rate was examined in ducks anesthetized with pentobarbital (50 mg/kg) Cyanide (400 μg) or nicotine (80 μg) was injected rapidly through a catheter placed in aorta about 2 cm above the aortic valves Stimulation of the peripheral chemoreceptors with cyanide and nicotine always elicited hyperventilation and tachycardia

The animals were then paralyzed with curare or suxamethonium chloride which abolished the change in heart rate following chemoreceptor stimulation with cyanide or nicotine suggesting that the observed increase in heart rate is secondary to stimulation of ventilation This conclusion was also supported by a temporal dispersion of

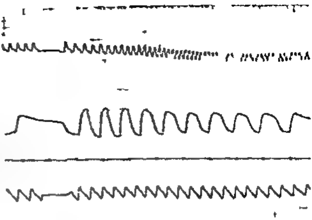

Fig 1 Changes in heart rate (upper tracing) and in ventilation (middle tracing) following injection of 80 µg nicotine The time of injection is marked by the flat portion of the heart rate tracing Ventilation was monitored with a thermistor placed right above the glottis. Note the delay in the heart rate response relative to the ventilatory response The lower tracing shows the absence of a heart rate response to injection of 80 µg nicotine when the animal had been paralyzed with curare 3 mg i v Time marking is 1 sec

the ventilatory response and the heart rate response to nicotine and cyanide injection The tachycardia was always delayed 1 to 3 sec relative to the increase in ventilation

In ducks the peripheral chemoreceptors and the baroreceptors were chronically denervated The heart rate in the non-diving animal increased 100 to 150 % due to denervation. Following denervation the heart rate did not change during a 1 min dive in the unanesthetized duck which could suggest that the peripheral chemoreceptors are essential in the mechanism of the diving bradycardia because other evidence indicates that the baroreceptors are not essential in the diving response (Johansen and Aakhus 1963 Folkow Nilsson and Yonce 1967)

Because stimulation of the peripheral chemoreceptors do not elicit bradycardia we must however conclude that they probably do not play a role in the development of the diving bradycardia. The absence of a diving bradycardia in the denervated ducks may indicate only that the new circulatory state caused by the baroreceptor denervation inhibits the diving response The pronounced effect of respiratory movements on heart rate may be of importance for the increase in heart rate immediately following the first breath after emersion from a dive

References

Folkow B N J Nilsson and L. R. Yonce *Acta physiol scand* 1967 *70* 347—361
Johansen K and T Aakhus *Amer J Physiol* 1963 *205* 1167—1171

Acta physiol scand. 1970. 79. 289—298

From King Gustaf V th Research Institute Stockholm and the Department of Geriatrics University of Uppsala Sweden

Some Characteristics of Lipolysis in Rabbit Adipose Tissue. Effects of Noradrenaline, ACTH, Theophylline and Prostaglandin E_1

By

HORACE MICHELI

Received 6 February 1969

Abstract

MICHELI, H *Some characteristics of lipolysis in rabbit adipose tissue Effects of noradrenaline, ACTH, theophylline and prostaglandin E_1* Acta physiol scand 1970 *79* 289—298

Perirenal adipose tissue from rabbits of different strains was incubated in vitro and the glycerol release determined to evaluate the lipolysis Noradrenaline was found to stimulate lipolysis but to a lower degree than ACTH From one strain of rabbits it was apparent that lighter (younger) rabbits were more responsive to noradrenaline than heavier (older) animals some of which were completely unresponsive to the catecholamine Theophylline as well as PGE_1 were ineffective when added alone to the incubation medium However, theophylline increased the glycerol release induced by noradrenaline and low concentrations of ACTH and PGE_1 inhibited lipolysis under these conditions The results suggest that there is a low rate of formation of cyclic AMP in rabbit adipose tissue under basal conditions of incubation while the hormonal stimulations might well operate by means of increasing the accumulation of cyclic AMP

In a recent study it was observed that nicotinic acid which is usually a potent inhibitor of mobilization of free fatty acids (FFA) from adipose tissue under various conditions was a poor inhibitor of ACTH induced stimulation of FFA mobilization in the rabbit (Boberg *et al* 1969 a) Also the stimulation of lipolysis in adipose tissue elicited by noradrenaline was not always inhibited by nicotinic acid (ibid) Furthermore nicotinic acid did not lower basal FFA levels in fed rabbits These findings were unexpected and it was decided to characterize the response of rabbit adipose tissue to various lipolytic and antilipolytic agents *in vitro* since this had not been done systematically (Rudman and Di Girolamo 1967) In a following paper the effects of nicotinic acid will be studied on noradrenaline and ACTH induced lipolysis in rabbit adipose tissue and compared to effects of prostaglandin E_1 (PGE_1) (Boberg *et al* 1969 b)

Material and methods

Male rabbits from different strains, weighing 1800 to 5000 g were used after a stay in the animal department for at least three days They had free access to food until sacrifice At 10 a m the rabbit was killed by ... ation by carotid art section Perirenal adipose tissue was ... saline and cut into pieces of 50—100 mg Three or four ... rirenal fat were put into in cubation flasks of glass in or ... The wet weight was deter mined by weighing the flasks before and after addition of the adipose tissue The flasks con tained 4 ml of a medium consisting of 2 % human albumin (AB Kabi, Stockholm, Sweden) and 0 1 % glucose in Krebs Ringer bicarbonate buffer, pH 7 4 After a preincubation period of the tissues in the medium for about 45 min at room temperature one ml of medium was taken for zero time analysis and various substances added in 0 1 ml of saline solutions, except in experiments including theophylline additions In such experiments, theophylline (ACO, Stockholm, Sweden) was dissolved in the med ... starting the incubation 2 ml of this sc ... tained only 2 ml The same procedure ... (Nor Exadrin conc ©, AB Astra Sode ... Uppsala Sweden) and stock solution of PGE_1 were dissolved at the last moment in 0 9 % saline Crystalline PGE_1 (supplied by Prof S Bergstrom, Karolinska institutet, Stockholm Sweden) was dissolved in 99 % ethanol and kept at —17° up to 4 months When PGE_1 was added in an experiment the same ...

Incubation time ... zymatically (Wielar ... rol 1 phosphate-deh ... all purchased from ... cording to Snedecor (1956)

Results

ffect of noradrenaline

e effect of noradrenaline was tested in two groups of rabbits The first group onsisted of 12 animals from different strains (Table I) and the second of 11 from one single strain (Table II) In this second group macroscopic differences existed in the amount and consistence of the perirenal fat, that of the lighter rabbits beeing scarce and smooth compared to that of the heavier With addition of 1 μg/ml of nor adrenaline to the incubation medium the glycerol release was increased significantly in all cases from the first group and in 8 of 11 from the second The 3 rabbits un responsive to noradrenaline had a body weight of more than 3000 g The lowest concentration of noradrenaline, 0 1 μg/ml, gave no significant increase of glycerol in the 5 rabbits tested in the first group, but in the second group a stimulatory effect was found in the 2 lighter rabbits, but not in the 2 above 3000 g Of the 5 animals tested with addition of 10 μg/ml to the medium only one showed a significantly higher response of lipolysis than with 1 μg/ml

Effect of ACTH

The effect of ACTH on lipolysis in rabbit adipose tissue from several strains is shown in Table III The concentration of 0 001 IU ACTH per ml stimulated lipolysis in the 4 rabbits shown in Table III but in a few other studies done later this dose was sometimes without any effect With 0 01 IU of ACTH lipolysis was further stimulated and in 5 rabbits of 8 0 1 IU caused a greater glycerol release than 0 01 IU The

TABLE I Effect of noradrenaline on glycerol release from adipose tissue incubated in vitro Peri renal adipose tissue was obtained from different strains of rabbits Mean values ± SEM and number of incubation flasks

Noradrenaline, μg/ml			0	0 1	1	10
No.	Body weight, g	Strain	Glycerol release, μmole/g wet wt/hr			
19	2300	N	0 25 ± 06 (7)	—	0 53 ± 05 (7) §	—
[illegible]	2300	B	0 40 ± 03 (7)	0 46 ± 04 (7)	0 71 ± 05 (7) ≠	—
33	2300	B	0 32 ± 04 (8)	—	0 99 ± 04 (8) ≠	—
41	2800	N	0 57 ± 04 (6)	0 69 ± 04 (6)	1 41 ± 05 (6) ≠	1 50 ± 09 (6) ≠
38	3000	N	0 33 ± 01 (10)	—	0 55 ± 01 (10) ≠	—
[illegible]	3200	N	0 32 ± 02 (8)	0 38 ± 10 (8)	0 54 ± 05 (8) §	—
48	3200	W	0 84 ± 03 (6)	—	1 27 ± 06 (6) ≠	1 29 ± 07 (6) ≠
14	3500	W	0 80 ± 12 (5)	0 90 ± 10 (6)	1 14 ± 07 (6) ⊖	1 21 ± 06 (5) ⊖
31	3600	N	0 57 ± 06 (8)	0 60 ± 07 (6)	1 26 ± 10 (8) ≠	—
35	3800	N	0 24 ± 02 (8)	—	0 37 ± 02 (8) ≠	—
42	4100	W	0 72 ± 09 (6)	—	2 36 ± 10 (6) ≠	—
43	4100	W	0 32 ± 04 (8)	—	0 99 ± 04 (8) ≠	—

Strains N = unknow origin B = blue black W = albinos
⊖ P < 0 05 § P < 0 01 and ≠ P < 0 001 for the statistical significance of the changes from basal

TABLE II Effect of noradrenaline on glycerol release from adipose tissue incubated in vitro Peri renal adipose tissue was obtained from one single strain of rabbit (light brown) Mean values ± SEM and number of incubation flasks

Noradrenaline, μg/ml		0	0 1	1	10
No	Body weight g	Glycerol release μmole/g wet wt/hr			
6	1800	0 50 ± 09 (6)	—	1 95 ± 09 (6) ≠	—
4	2000	1 62 ± 07 (7)	—	2 74 ± 08 (6) ≠	—
2	2200	1 48 ± 14 (4)	1 94 ± 09 (4) ⊖	3 08 ± 13 (4) ≠	—
9	2300	0 96 ± 04 (7)	1 51 ± 08 (7) ≠	1 83 ± 04 (7) ≠	2 18 ± 04 (7) ≠
7	2500	0 88 ± 04 (7)	—	2 13 ± 06 (7) ≠	—
10	2600	0 23 ± 05 (7)	—	1 53 ± 11 (7) ≠	—
23	3100	0 71 ± 12 (7)	—	0 94 ± 19 (7)	—
13	3500	1 05 ± 08 (6)	0 94 ± 04 (6)	1 07 ± 03 (6)	1 08 ± 03 (6)
39	3800	0 29 ± 02 (8)	—	0 39 ± 03 (8) ⊖	—
1	4000	0 50 ± 05 (4)	0 50 ± 03 (4)	0 49 ± 05 (4)	—
3	5000	0 75 ± 04 (6)	—	1 04 ± 08 (6) §	—

For symbols see Table I

TABLE III Effect of ACTH on glycerol release from adipose tissue incubated in vitro Perirenal adipose tissue was obtained from different strains of rabbits Mean values ± SEM and number of incubation flasks

ACTH, I U /ml		0	0 001	0 01	0 1	1
No	Body weight, g	Glycerol release, μmole/g/hr				
34	200	0 54 ± 06 (8)	1 24 ± 05 (8)	2 70 ± 04 (8)	3 01 ± 06 (8)	3 37 ± 05 (8)
8	2500	*1 69 ± 12 (9)*	*3 25 ± 13 (9)*	*5 28 ± 18 (7)*	*6 21 ± 24 (8)*	*7 38 ± 28 (8)*
28	2850	1 39 ± *12 (9)*	—	2 78 ± 12 (9)	3 20 ± 06 (7)	3 34 ± 10 (8)
12	3500	1 35 ± 15 (7)	1 95 ± 40 (7)	2 49 ± 50 (7)	3 07 ± 44 (7)	3 29 ± 49 (7)
15	*3500*	*1 08 ± 11 (9)*	*1 75 ± 09 (8)*	3 92 ± 33 (7)	4 82 ± 31 (7)	5 31 ± 50 (8)
30	3500	0 38 ± 03 (7)	—	1 72 ± 14 (7)	1 72 ± 05 (7)	1 97 ± 17 (7)
49	3700	*0 49 ± 08 (6)*	—	3 07 ± 10 (6)	—	4 77 ± 17 (6)
42	4100	0 32 ± 05 (7)	—	2 40 ± 03 (7)	2 79 ± 12 (7)	2 85 ± 09 (7)
43	4100	0 72 ± 09 (6)	—	3 39 ± 13 (6)	3 46 ± 20 (5)	3 99 ± 10 (6)

IV Effect of theophylline on glycerol release from rabbit adipose tissue incubated in vitro Perirenal adipose tissue was obtained from different strains of rabbits Mean values ± SEM and number of incubation flasks

Theophylline, Molar		0	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}	2.5×10^{-2}
No	Body weight, g	Glycerol release, μmole/g/hr					
[illegible]	2150	48 ± 06 (9)	51 ± [illegible] (8)	—	—	—	—
19	*2300*	*25 ± 06 (7)*	*32 ± 05 (7)*	—	—	—	—
32	2300	40 ± 03 (7)	—	35 ± 03 (7)	—	52 ± 03 (7)	—
24	2850	43 ± 07 (7)	36 ± 04 (7)	48 ± 05 (7)	48 ± 04 (7)	50 ± 06 (7)	43 ± 11 (7)
38	3000	33 ± 01 (10)	—	—	—	35 ± 01 (10)	—
27	3100	75 ± 09 (8)	—	83 ± 13 (5)	—	73 ± 07 (9)	—
22	3200	27 ± 01 (7)	29 ± 01 (7)	24 ± 04 (7)	32 ± 02 (7)	36 ± 03 (7) ⊕	36 ± 03 (7) ⊕
29	3200	32 ± 02 (8)	—	34 ± 02 (8)	—	37 ± 01 (8)	—
48	3200	[illegible] ± 03 (6)	—	94 ± 06 (6)	—	—	—
47	3300	53 ± 01 (6)	—	60 ± 02 (6)	—	—	—
31	3600	57 ± 04 (8)	—	52 ± 04 (8)	—	55 ± 05 (8)	—
35	3800	24 ± 01 (8)	—	25 ± 03 (8)	28 ± 03 (8)	39 ± 03 (8) ⊕	—

For symbols, see Table I

TABLE V Effect of theophylline on noradrenaline stimulated glycerol release from rabbit perirenal adipose tissue incubated in vitro and additional effect of prostaglandin E[1] (PGE_1) Mean values ± SEM and number of incubation flasks

Noradrenaline, μg/ml		0	1	1	1
Theophylline, Molar		0	[illegible]	10 *	10 *
PGE_1 μg/ml		0	0	0	1
No	Body weight, g	Glycerol release, μmole/g/hr			
37	2000	0 33 ± 03 (8)	—	1 98 ± 08 (8)	0 34 ± 02 (8)
32	2300	0 40 ± 03 (7)	0 71 ± 05 (7)	1 47 ± 07 (7)	—
33	2300	0 32 ± 04 (8)	0 99 ± 04 (8)	1 69 ± 03 (8)	0 59 ± 06 (8)
19	2300	0 25 ± 06 (7)	0 53 ± 05 (7)	1 33 ± 10 (7)	0 33 ± 05 (7)
36	2500	0 65 ± 03 (8)	—	1 24 ± 03 (8)	0 61 ± 04 (8)
38	3000	0 33 ± 01 (10)	0 55 ± 01 (10)	0 74 ± 02 (10)*	—
23	3150	0 71 ± 12 (7)	0 94 ± 19 (7)	1 69 ± 12 (7)	—
29	3200	0 32 ± 02 (8)	0 84 ± 05 (8)	1 05 ± 07 (8)	—
48	3200	0 84 ± 03 (6)	1 27 ± 06 (6)	2 21 ± 08 (6)	—
25	3350	—	0 84 ± 05 (7)	1 39 ± 10 (7)	0 64 ± 04 (7)**
[illegible]	3600	0 57 ± 04 (8)	1 26 ± 06 (8)	2 88 ± 06 (8)	—
39	3800	0 29 ± 02 (8)	0 39 ± 03 (8)	0 61 ± 03 (8)	0 30 ± 02 (8)

* 10 ³ Molar theophylline
** 0 1 μg/ml PGE_1

highest concentration tested was for practical reasons one IU/ml Three rabbits of 8 responded with an enhanced lipolysis when ACTH was increased from 0 1 to one IU

Effect of theophylline

Theophylline was added to the medium in concentrations from 5×10^{-4} to $2\ 5 \times 10^{-2}$ M (Table IV) There was no effect at all on lipolysis in 6 out of 8 rabbits at 10^{-3} M and among all experiments only a very weak stimulating effect was found in 3 of them

On the other hand theophylline had always a powerful synergistic effect on noradrenaline induced stimulation of glycerol release (Table V) Under these conditions 10^{-3} M theophylline, which concentration was almost completely devoid of activity when added under basal conditions (Table IV) in all rabbits significantly stimulated lipolysis

In a similar way theophylline markedly enhanced lipolysis in the presence

TABLE VI Effect of theophylline on ACTH stimulated glycerol release from rabbit perirenal adipose tissue incubated in vitro and additional effect of prostaglandin E_1 (PGE_1) Mean values ± SEM and number of incubation flasks

ACTH, I U/ml		0	0 0025	0 0025	0 0025	0 01	0 01	0 01	1
Theophylline, Molar		0	0	10^{3}	10^{3}	0	10^{3}	10^{3}	0
PGE_1, μg/ml		0	0	0	1	0	0	1	0
No	Body weight, g	Glycerol release, μmole/g/hr							
27	3100	0 75 ± 09 (8)	—	—	—	2 85 ± 25 (9)	2 78 ± [illegible] (9)*	2 61 ± 15 (8)*	—
40	3100	1 69 ± 18 (6)	—	—	—	4 15 ± 25 (6)	4 19 ± 25 (6)	3 60 ± 25 (6)	—
46	3200	0 67 ± 11 (5)	0 65 ± 07 (5)	1 90 ± 14 (6)	—	2 43 ± 13 (6)	3 17 ± 11 (6)	—	3 93 ± 19 (6)
49	3700	0 49 ± 08 (6)	1 11 ± 10 (6)	2 57 ± 04 (6)	0 61 ± 03 (6)	—	—	—	4 77 ± 17 (6)
[illegible]	3800	0 29 ± 02 (8)	0 72 ± 05 (8)	1 63 ± 03 (8)	0 85 ± 05 (8)	—	—	—	—

10^{3} Molar theophylline

and even in itself ineffective concentrations of ACTH (Table VI) At a higher concentration of ACTH 0 01 IU/ml, which dose gave submaximal stimulation of lipolysis in 5 of the 8 rabbits presented in Table III the glycerol release was not increased by theophylline addition in 2 of 3 rabbits

Effect of PGE_1

PGE_1 at concentrations from 0 01 to 10 μg/ml did not decrease the basal glycerol release (Table VII) In one rabbit there was a slight increase with 10 μg But when lipolysis was stimulated by noradrenaline alone (Table VII) as well as in combination with theophylline (Table V), PGE_1 significantly suppressed the increased lipolytic rate

PGE_1 also inhibited the stimulation induced by a low concentration of ACTH 0 0025 IU/ml while that induced by the higher concentration 0 01 IU/ml was inhibited only in 1 of 8 rabbits (Table VIII) It is obvious that 0 01 IU/ml of ACTH did not cause the maximal lipolytic stimulation in two of these rabbits (No 28 and

TABLE VII Effect of prostaglandin E_1 (PGE_1) on spontaneous and on noradrenaline stimulated glycerol release from rabbit perirenal adipose tissue incubated in vitro Mean values ± SEM and *number of incubation flasks*

PGE_1, µg/ml		0	0 01	0 1	1	10	0	1
Noradrenaline, µg/ml		0	0	[illegible]	0	0	1	1
No	Body weight g	Glycerol release, µmole/g/hr						
6	1800	0 50± 09 (6)	—	—	0 46± 07 (6)	—	1 95± 09 (6)	0 75± 11 (6)
4	2000	1 62± 07 (7)	—	—	1 67± 15 (6)	—	—	—
7	2500	0 88± 04 (7)	—	—	0 98± 09 (7)	—	2 13± 06 (7)	1 08± [illegible] (7)
10	2650	0 23± 05 (7)	0 37± 06 (7)	0 31± 11 (7)	0 36± 09 (7)	—	1 52± 11 (7)	0.50±07 (7)
41	2800	0 57± 04 (5)	—	—	—	—	1 41± 05 (5)	0 59± 04 (5)
48	3200	0 84± 03 (6)	—	—	0 85± 04 (6)	0 77± 04 (6)	—	—
50	3500	0 70± 04 (6)	—	—	—	0 68± 02 (6)	1 10± 02 (6)	0 88± 02 (6)
51	3700	0 38± 02 (6)	—	—	—	0.56± 04⊕ (6)	1 07± 07 (6)	0 65± 02 (6)
20	4500	0 44± 05 (10)	0 47± 02 (10)	0 44± 03 (8)	0 44± 02 (9)	—	—	—
3	5000	0 75± 04 (6)	—	—	0 69± 08 (6)	—	—	—

For symbols, see Table I

49) where PGE_1 had no inhibitory action In all 4 rabbits stimulated with 0 1 IU of ACTH PGE_1 was without effect on lipolysis PGE_1 was in the case of ACTH as with noradrenaline effective in reducing the increase in glycerol release obtained when also theophylline was added to the incubation medium (Table VI)

Discussion

It has previously been found by Rudman *et al* (1963) that neither adrenaline nor noradrenaline in doses as high as 100 µg/ml incubation medium stimulated lipolysis in rabbit adipose tissue *in vitro* We found here that noradrenaline was a potent

TABLE VIII Effect of prostaglandin E_1 (PGE_1) on ACTH stimulated glycerol release from rabbit perirenal adipose tissue incubated in vitro Mean values ± SEM and number of incubation flasks

ACTH I U/ml		0	0 0025	0 0025	0 01	0 01	0 1	0 1
PGE_1 µg/ml		0	0	1	0	1	0	1
No	Body weight g	Glycerol release, µmole/g/hr						
16	3750	1 33 ± 14 (10)	—	—	—	—	3 97 ± 15 (10)	3 74 ± 15 (7)
17	3850	1 17 ± 11 (7)	—	—	—	—	4 62 ± 14 (7)	4 25 ± 11 (7)
28	2850	1 39 ± 12 (9)	—	—	2 78 ± 12 (9)	2 81 ± 10 (9)	3 20 ± 06 (7)	3 08 ± 07 (6)
30	3500	0 38 ± 03 (7)	—	—	1 72 ± 14 (7)	1 64 ± 09 (7)	1 72 ± 05 (7)	1 69 ± 05 (6)
21	2200	0 72 ± 04 (6)	—	—	1 47 ± 08 (6)	1 19 ± 09⊕	—	—
36	2500	0 65 ± 03 (8)	1 39 ± 10 (8)	0 81 ± 04≠ (8)	2 28 ± 11 (8)	1 94 ± 13	—	—
7	2000	0 33 ± 02 (8)	0 91 ± 04 (8)	0 30 ± 03≠ (8)	1 76 ± 06 (8)	1 65 ± 04 (8)	—	—
19	3700	0 49 ± 08 (6)	1 11 ± 10 (6)	0 40 ± [illegible]≠ (6)	3 07 ± 10 (6)	3 08 ± 07 (6)	4 77 ± 17* (6)	—

For symbols see Table I
* 1 I U /ml ACTH

lipolytic stimulator except in one strain of rabbit although ACTH elicited a still greater stimulation of lipolysis The difference in results is difficult to explain and may be related to factors such as strain and age In one of our strains the lipolytic response to noradrenaline clearly declined with increase in body weight (Table II)

We have also seen that *in vivo* noradrenaline perfusion causes greater increase in plasma FFA in rabbits of lighter than in heavier weights and this effect disappears within ten minutes after the perfusion is withdrawn (Boberg *et al* 1969 a) Furthermore Svedmyr (1966) and Dzedin *et al* (1968) have observed that catecholamines increase the concentration of FFA in plasma in rabbit The mean body weight of their rabbits was 2 0 kg for the adrenaline and 2 6 kg for the noradrenaline experiments On the other hand Rudman *et al* (1963) stated that subcutaneous injection of cathecholamines did not cause any change in serum FFA concentration in the in

tact rabbit 1 5 hrs after injection In their study the rabbits had a body weight be tween 3 0 and 4 5 kg Thus the difference in weight of the rabbits between the above studies reflecting possibly differences in age in spite of using other strains might be a major reason of these contradictory results *in vivo* However Svedmyr (1966) showed that the metabolic action of catecholamines (on FFA) is short in rabbit and therefore might have been missed by Rudman *et al* (1963) in their experiments *in vivo*

The results obtained with noradrenaline *in vitro* in one strain (Table II) suggest that age may be of importance for the lipolytic response to noradrenaline in rabbit adipose tissue *in vitro* In rat such a decrease of the response with aging is well known (Jelinkova and Hruza 1964) In man this was not apparent from a series of patients between 13 and 76 years of age studied in this laboratory (Micheli *et al* 1969) but a tendency to get higher response in children and young adults than in older subjects has been found by others (Bjorntorp *et al* 1969 Mosinger *et al* 1965)

Addition of theophylline to rabbit adipose tissue in vitro had a very slight effect if any on the glycerol release This is in contrast to many other species Theophylline stimulates lipolysis in vitro in rat (Hynie *et al* 1966) and in human adipose tissue (Carlson *et al* 1969) However injected into rabbit theophylline has been found to increase the plasma FFA (Paoletti *et al* 1967)

Those previous results contrast with our negative findings in rabbit adipose tissue incubated with theophylline alone However theophylline augmented both nor adrenaline and ACTH stimulated lipolysis which indicated that theophylline can affect the process of lipolysis in rabbit adipose tissue as soon as it is hormonally ac tivated An explanation to the low effect of theophylline on basal lipolysis would be that there is a very low rate of formation of cyclic AMP under basal conditions in the rabbit adipose tissue

In this connection the activity of PGE_1 in rabbit adipose tissue is of great interest the parallelism with theophylline activity is striking no effect of PGE_1 when added alone to the fat tissue and potent inhibitory activity as soon as noradrenaline or low concentrations of ACTH are stimulating glycerol release It is assumed that PGE_1 inhibits lipolysis in adipose tissue by inhibiting accumulation of cyclic AMP (Butcher and Baird 1968) When both PGE_1 and theophylline are devoid of their respective effects as we have found here in the basal state this could be explained on a common basis if there is a low rate of accumulation of cyclic AMP However when lipolysis was stimulated by noradrenaline or ACTH at low doses both theophylline and PGE were active the first stimulating and the latter inhibiting lipolysis This suggests that the hormonal stimulation of lipolysis was mediated by increasing the rate of forma tion of cyclic AMP

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References

BJÖRNTORP, P, M KARLSSON and A HOVDEN, Quantitative aspects on lipid mobilization in human adipose tissue studied in vitro *Acta med scand* 1969 In press

BOBERG, J, H MICHELI and L RAMMER, Effect of nicotinic acid on ACTH and noradrenaline stimulated lipolysis in the rabbit I In vivo studies *Acta physiol scand* 1970 *79* 153—157

BOBERG, J, H MICHELI and L RAMMER, Effect of nicotinic acid on ACTH and noradrenaline stimulated lipolysis in the rabbit II In vitro studies including comparison with prostaglandin E_1 *Acta physiol scand* 1970 b *79* 299—304

BUTCHER, R W and C E BAIRD, Effects of prostaglandins on adenosine 3',5'-monophosphate levels in fat and other tissues *J biol Chem* 1968 *243* 1713—1717

CARLSON, L A, D HALLBERG and H MICHELI, Quantitative studies on the lipolytic response of human subcutaneous and omental adipose tissue to noradrenaline and theophylline *Acta med scand* 1969 *135* In press

D[illegible]

H[illegible]

153 90—96

[illegible]

Press, New York 1967 pp 35—117

[illegible]LDMAN, D, S J BROWN and M F MALKIN, Adipokinetic actions of adrenocorticotropin thyroid-stimulating hormone, vasopressin, α and β melanocyte-stimulating hormones, fraction H, epinephrine and norepinephrine in the rabbit, guinea pig hamster, rat, pig and dog *Endocrinology* 1963 *72* 527—543

SNEDECOR, G W, *Statistical Methods* The Iowa State Univ Press Ames, Iowa 1956

SVEDMYR, N, The influence of thyroxine treatment and thyroidectomy on the calorigenic and some other metabolic effects of adrenaline and noradrenaline in experiments on fasted rabbits *Acta physiol scand* 1966 *66* 257—268

WIELAND, O, Eine enzymatische Methode zur Bestimmung von Glycerin *Biochem Z* 1957 *329* 313—319

Acta physiol. scand. 1970. 79. 299—304

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Effect of Nicotinic Acid on ACTH and Noradrenaline Stimulated Lipolysis in the Rabbit. II. In Vitro Studies Including Comparison with Prostaglandin E_1

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Abstract

BOBERG, J., H. MICHELI and L. RAMMER *Effect of nicotinic acid on ACTH and noradrenaline stimulated lipolysis in the rabbit II In vitro studies including comparison with prostaglandin E_1* Acta physiol scand 1970 79 299—304

Perirenal adipose tissue from male rabbits was incubated in vitro and the glycerol release followed as a measure of lipolysis. Addition of nicotinic acid to the medium did not change the basal release Nicotinic acid inhibited the noradrenaline stimulated lipolysis only in 3 of 8 rabbits while PGE_1 under the same conditions was inhibitory in 6 of 6 rabbits Nicotinic acid only inhibited lipolysis stimulated by ACTH at low doses in one of seven experiments In comparison to PGE_1 nicotinic acid was found to be a weaker inhibitor of lipolysis in rabbit adipose tissue stimulated in various ways in vitro These findings suggest that the previously seen weak properties of nicotinic acid as a FFA lowering compound in rabbits may be explained by factors within adipose tissue

Some characteristics of lipolysis from adipose tissue in rabbit in vivo (Boberg *et al* 1969) and in vitro (Micheli 1969) have been described before In vivo nicotinic acid injected to rabbits did not change the basal arterial concentration of plasma free fatty acids (FFA), but inhibited in some animals the noradrenaline induced increase of FFA mobilization and suppressed the increase of plasma FFA concentration caused by injection of low doses of ACTH In vitro noradrenaline and ACTH stimulated lipolysis in rabbit adipose tissue The lipolysis caused by low doses of these drugs was further increased by theophylline However theophylline per se which stimulates lipolysis in rat and human adipose tissue had no effect on basal lipolysis in the rabbit Prostaglandin E_1 (PGE_1) was found to be an effective inhibitor of noradrenaline and ACTH stimulated lipolysis in vitro, but without eff on basal lipolysis in the rabbit (Micheli 1969)

TABLE I Effect of nicotinic acid on basal, on noradrenaline and on noradrenaline plus theophylline in vitro and effect of PGE_1 on noradrenaline stimulated glycerol release Mean values ± ments

		(A)	(B)	(C)	(D)
Nicotinic acid, μg/ml		0	1	10	0
Prostaglandin, E_1, μg/ml		0	0	0	0
Noradrenaline, μg/ml		0	0	0	1
Theophylline, Molar		0	0	0	0
No	Body weight, g				
1	4000	0 50 ± 05 (4)	0 48 ± 03 (4)	—	—
2	*2200*	*1 48 ± 14 (4)*	*1 71 ± 13 (4)*	—	*3 08 ± 13 (4)*
3	5000	0 75 ± 04 (6)	0 62 ± 09 (6)	—	—
4	2000	1 62 ± 07 (7)	1 54 ± 17 (5)	—	2 74 ± 08 (4)
7	2500	0 88 ± 04 (7)	0 94 ± 09 (7)	—	2 13 ± 06 (7)
50	3500	0 70 ± 04 (6)	—	0 70 ± 03 (6)	1 10 ± 02 (6)
51	3700	0 38 ± 02 (6)	—	0 51 ± 02 (6)	1 07 ± 07 (6)
52	3000	0 29 ± 02 (6)	—	—	0 61 ± 04 (5)
53	2500	0 82 ± 06 (6)	—	—	1 25 ± 05 (6)
54	2550	0 54 ± 02 (6)	—	—	1 48 ± 06 (6)

The purpose of this work was to determine the effect of nicotinic acid on lipolysis in rabbit adipose tissue in vitro and to compare the inhibitory properties of nicotinic acid and PGE_1

Material and methods

Male rabbits of different strains weighing between 2000 and 5000 g were used After at least 3 days stay in the animal department with free access to food the animals were killed by a blow on the nape followed by exsanguination Perirenal adipose tissue was excised and cut into 50—100 mg pieces and three to four pieces were put into incubation flasks containing 4 ml of a Krebs-Ringer buffer with 2 per cent albumin and 0 1 per cent glucose as described in detail elsewhere (Micheli 1969 After a preincubation time of around 45 min at room temperature the flasks were incubated at 37 C for one hr and aliquots were taken off at 0 and 60 min for determination of glycerol

Lipolysis was expressed as release of glycerol (μmole/g wet weight/hr) The values given for each experiment are the mean ± s e m of 4—10 incubation flasks The drugs tested were added to the incubation medium at zero time

Drugs used were theophylline and nic ... adrenaline (Nor-exadrin conc® Astra Sodertalje ... Uppsala Sweden) and prostaglandin E_1 kindly ... Sweden) Solutions and additions were as describe ... ods were used according to Snedecor 1956

Results

Basal glycerol release varied between 0 29 and 1 62 μmole/g/hr (Table I) This release was not significantly changed by the addition of nicotinic acid 1 μg/ml (Table

stimulated glycerol release (µmole/g wet weight/ hr) from rabbit perirenal adipose tissue incubated SEM and number of incubation flasks See Table II for P values of the difference between experi-

(E)	(F)	(G)	(H)	(J)
1	10	0	0	1
[illegible]	0	1	0	0
1	1	1	0 1	0 1
0	0	0	10[illegible]	10[illegible]

Glycerol release, µmole/g/hr

—	—	—	—	—
2 79 ± 11 (5)	—	—	—	—
—	—	—	—	—
2 15 ± 08 (6)	—	—	—	—
1 54 ± 06 (7)	—	1 08 ± 08 (7)	—	—
1 22 ± [illegible] (6)	1 07 ± 06 (6)	0 88 ± 02 (6)	—	—
0 96 ± 03 (6)	0 97 ± 02 (6)	0 65 ± 02 (6)	—	—
0 53 ± 03 (6)	0 55 ± 06 (6)	0 34 ± 03 (6)	0 40 ± 05 (6)	0 29 ± 03 (6)
1 20 ± 03 (6)	1 17 ± 05 (6)	0 91 ± 03 (6)	2 07 ± 06 (6)	1 87 ± 02 (5)
1 22 ± 09 (6)	1 25 ± 07 (6)	0 96 ± 02 (6)	1 38 ± 05 (6)	1 04 ± 05 (6)

I and II) In one rabbit basal lipolysis was unchanged even by 10 µg/ml of nicotinic acid, while in another rabbit basal release was increased by this concentration of nicotinic acid

In all experiments given in Table I noradrenaline increased basal lipolysis This increase was *significantly inhibited by nicotinic acid, 1 µg/ml, in 3 of 8 rabbits and* stimulated in one There was no difference in lipolysis when the concentration of nicotinic acid was increased to 10 µg/ml except for rabbit number 50 where the slight increase of lipolysis caused by the low nicotinic acid concentration disappeared In the rabbits tested with both PGE_1 and nicotinic acid on nordarenaline stimulated lipolysis PGE_1 caused a highly significant inhibition (45 to 85 per cent) in all the animals while significant inhibition with nicotinic acid only occured in 3 of 6 rabbits The stimulation of lipolysis by noradrenaline (N [illegible] 0 1 µg/ml) plus theophylline was inhibited by nicotinic acid in all three rabbits studied but significantly so only in two

Table III gives the results of the effect of nicotinic acid on the ACTH stimulated lipolysis in rabbit adipose tissue Basal glycerol release in these rabbits was between 0 38 and 1 48 µmol/g/hr The lowest concentration of ACTH, 0 0025 IU/ml increased lipolysis significantly in one of three rabbits ACTH concentrations of 0 01 0 1 and 1 0 IU/ml increased lipolysis in all animals studied Except for one rab (no 21), nicotinic acid had no significant inhibitory effect on the ACTH st

TABLE II P values of the statistical difference between the experiments in Table I Letters refer to the columns in Table I ns = not significant

Compared exper.		B—A	E—D	F—D	C—D	J—H
Rabbit no	1	ns	—	—	—	—
	2	ns	ns	—	—	—
	3	ns	—	—	—	—
	4	ns	<0 001	—	—	—
	7	ns	<0 001	—	<0 001	—
	50	—	<0 01*	ns	<0 001	—
	51	—	ns	ns	<0 001	—
	52	—	ns	ns	<0 001	ns
	53	—	ns	ns	<0 001	<0 05
	54	—	<0 05	<0 05	<0 001	<0 001

* the effect of nicotinic acid was a stimulation

TABLE III Effect of nicotinic acid on ACTH stimulated glycerol release from rabbit perirenal adipose tissue Mean values ± SEM and number of incubation flasks

ACTH, I U /ml		0	0 0025	0 0025	0 01	0 01	0 1	0 1	1
Nicotinic acid, μg/ml		0	0	1	0	1	0	1	0
	Body weight, g	Glycerol release, μmole/g/hr							
16	3750	1 33± 14 (10)	—	—	—	—	3 97± 15 (10)	3 68± 10 (8)	—
17	3850	1 17± 11 (7)	—	—	—	—	4 62± 14 (7)	4 44± 14 (7)	—
28	2850	1 39± 12 (9)	—	—	2 78± 12 (9)	2 61± 10 (9)*	3 20± 06 (7)	3 48± 14 (6)*	—
30	3500	0 38± 03 (7)	—	—	1 72± 14 (7)	1 57± 09 (7)	1 72± 05 (7)	1 64± 10 (7)	—
21	2200	0 72± 04 (6)	—	—	1 47± 08 (6)	1 20± 07 (6)**	—	—	—
45	3000	1 48± 07 (6)	1 63± 09 (6)	1 72± 09 (6)	2 76± 10 (6)	2 93± 11 (6)	—	—	—
46	3200	0 67± 11 (5)	0 65± 07 (5)	0 64± 08 (5)	2 43± 13 (6)	2 38± 12 (6)	—	—	3 93± [illegible] (6)
49	3700	0 49± 08 (6)	1 11± 10 (6)	0 97± 07 (6)	3 07± 10 (6)	3 14± 09 (6)	—	—	4 77± 17 (6)

* 0 1 μg/ml nicotinic acid instead of 1

** p<0 05 significance of nicotinic acid inhibition

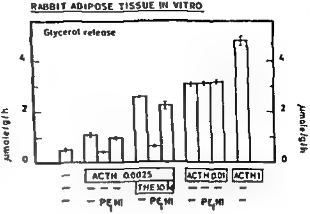

Fig 1 Comparison of effects of nicotinic acid (NI, 1 µg per ml) and of prostaglandin E_1 (PE_1 1 µg per ml) on ACTH and ACTH plus theophylline stimulated glycerol release from rabbit perirenal adipose tissue incubated in vitro (Rabbit number 49) Concentrations of ACTH are indicated in IU per ml Mean ± SEM from eight incubation flasks are given

lipolysis (Table III and Fig 1) In this context it shall be stressed that even submaximal stimulation of lipolysis (*cf* rabbit no 46 and 49) was not inhibited by nicotinic acid

A comparison between nicotinic acid and PGE_1 as inhibitors to ACTH stimulated lipolysis in adipose tissue from one rabbit is given in Fig 1 The submaximal stimulation of lipolysis caused by ACTH, 0 0025 IU/ml, was completely inhibited by PGE_1 but not significantly changed by nicotinic acid Lipolysis induced by a higher ACTH concentration in the medium (0 01 IU/ml), but still submaximal, was not decreased by either PGE_1 or nicotinic acid

Theophylline synergistically augmented the lipolysis induced by low concentrations of ACTH (Fig 1) Nicotinic acid slightly inhibited this lipolysis ($P<0\,05$), while PGE_1 almost completely blocked stimulation of lipolysis caused by theophylline plus ACTH

Discussion

One of the reasons for undertaking this study was that in our previous experiments (Boberg *et al* 1969) nicotinic acid given intravenously to rabbits either did not change or lowered only slightly the concentration of FFA in plasma This finding was contradictory to earlier results with nicotinic acid in man, rat and dog (Carlson and Oro 1962 *cf* Carlson 1965) where nicotinic acid always had a prompt and pronounced lowering effect on plasma FFA levels under basal conditions as well as when the FFA levels had been raised with agents stimulating lipolysis The FFA lowering action of nicotinic acid has been ascribed to a direct inhibition of lipolysis in adipose tissue (Carlson 1963 Eaton 1963 *cf* Carlson 1965) The different effect of nicotinic acid in rabbit compared to other species could of course be due to factors within adipose tissue or to factors outside this tissue

In the present work in vitro the basal glycerol release was not lowered by nicotinic acid Furthermore the ACTH enhanced lipolysis was inhibited only in one experiment even though doses causing submaximal stimulation were used The maxi

enhancement of glycerol output elicited by noradrenaline was inhibited inconstantly by nicotinic acid

These findings concerning the effect of nicotinic acid on isolated rabbit adipose tissue incubated in vitro differ from the consistent and pronounced inhibitory actions of nicotinic acid on rat adipose tissue In that tissue nicotinic acid at a concentration of 0 02—0 04 μg per ml under incubation conditions identical to those used here always lowers basal and submaximally stimulated glycerol release by 50 per cent (Carlson, personal communication) In the present study not even 10 μg of nicotinic acid had any inhibitory effect on basal lipolysis The present findings with rabbit adipose tissue strongly suggest that our in vivo results are best explained by factors within adipose tissue The nature of these factors is not known The comparison of the antilipolytic effects of PGE_1 and of nicotinic acid is of interest Both substances had no action in the basal state, in contrast with observations in human and rat tissue Both PGE_1 and nicotinic acid have been shown under certain conditions to prevent the accumulation of cyclic AMP in rat adipose tissue and this property has been offered as an explanation for their antilipolytic actions (Butcher *et al* 1968) The present finding that these compounds have no antilipolytic effect on rabbit adipose tissue is then in accordance with the previous suggestion that the rate of formation of cyclic AMP is low under basal conditions in rabbit adipose tissue (Micheli 1969)

When the glycerol output was stimulated by noradrenaline or by low doses of ACTH PGE_1 inhibited lipolysis more potently than did nicotinic acid but higher nd still submaximal doses of ACTH were not antagonized even by PGE_1 From this ne could say nothing more than there was a quantitative difference between the inhibitory action of both substances which may or may not be due to different mechanisms of action

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References

BOBERG J H MICHELI and L RAMMER Effect of nicotinic acid on ACTH and on noradrenaline stimulated lipolysis in the rabbit I In vivo studies *Acta physiol scand* 1970 79 153—157

BUTCHER R W C E BAIRD and E W SUTHERLAND Effects of lipolytic and antilipolytic substances on adenosine 3 5 monophosphate in isolated fat cells *J biol Chem* 1968 243 1705—1712

CARLSON L A Studies on the effect of nicotinic acid on catecholamine stimulated lipolysis [illegible] *scand* 1963 *173* 719—722

[illegible] [mobi]lization of free fatty acids from adipose tissue *Ann* [illegible]

[illegible] nicotinic acid on the plasma free fatty acids Demonstration of a metabolic type of sympathicolysis *Acta med scand* 1962 *172* 641—645

EATON R P In vitro inhibition of the fat mobilizing action of norepinephrine *Proc Soc exp Biol* (N Y) 1963 *114* 599—600

MICHELI H, Some characteristics of lipolysis in rabbit adipose tissue effect of noradrenaline ACTH theophylline and prostaglandin E_1 *Acta physiol scand* 1970 79 289—298

SNEDECOR G W *Statistical methods* The Iowa State College Press Ames Iowa 1956

Acta physiol. scand. 1970. 79. 305—320

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Excitation and Impulse Conduction in the Human Fetal Heart

By

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Abstract

GENNSER, G and E NILSSON *Excitation and impulse conduction in the human fetal heart* Acta physiol scand 1970 *79* 305—320

Intracellular and bipolar surface electrodes were used to record electrical activity from isolated hearts of human fetuses at midgestation (17—24 weeks) Action potential characteristics for cells in the sinus node, the atrium, the AV-node, and the ventricle are described *Diastolic* depolarizations and subthreshold oscillations indicating latent pacemaker activity were found in atrial cells but not in ventricle cells Resting membrane potential in ventricle cells was −81.4 mV at 37° C and an external potassium concentration of 4 mM With increasing external potassium concentrations, the resting membrane potential increased, within the *range* 8—64 mM the change was 51.2 mV per tenfold increase in potassium concentration In 0.5 mM potassium solution, the amplitude of the resting membrane potential *declined in* ventricle cells and spontaneous action potentials appeared Within the temperature range 37—19° C, a decrease in temperature increased the resting membrane potential 0.9 mV per °C Conduction velocity in both atrium and ventricle was 0.4—0.7 m/sec In ventricle, the conduction velocity decreased with increasing stimulation frequency within the *range studied* (12—240 stim /min) In atrium, the conduction velocity decreased both at frequencies above 100/min and at very low frequencies The conduction velocity *through the AV node* was approximately 0.01 m/sec and decreased with increasing stimulation frequency above 100 stim /min

Relatively little is known about the electrophysiology of prenatal mammalian hearts It has been demonstrated that the spontaneous contraction frequency in isolated parts of the prenatal rat heart at midgestation is greatest in the sinus venosus region and decreases along a cranio-caudal line (Hall 1954) Furthermore, in a recent study concerning prenatal development of the rat heart, diastolic depolarizations were found not only in the sinus venosus, but also in both atria, indicating a widely distributed latent pacemaker activity (Couch *et al* 1969) This atrial diastolic depolarization disappeared during the prenatal development Thus, the ability to generate spontaneous action potentials seems to be a property inherent in most embryological heart cells which is normally lost during differentiation, except in those heart cells that function as pacemaker sites in the adult heart The amplitude

of the resting membrane potential and the upstroke velocity of the action potential increased during the later half of the gestation in the fetal rat heart (Couch *et al* 1969)

Earlier studies on human fetal hearts have been concerned with the morphology of the conduction system (Robb *et al* 1948, Truex *et al* 1959), the influence of calcium (Lloyd 1929) and adrenaline, acetylcholine (Garrey and Townsend 1948 Baker 1953) on the mechanical activity of the heart In a recent study it was shown that the mechanical activity, measured in terms of the active state parameter, in human fetal papillary muscles was dependent upon muscle length and contraction rate in much the same way as in similar preparations from various adult mammalian hearts (Gennser and Nilsson 1968) The epicardial excitation pattern in isolated perfused human fetal hearts has been studied (Durrer *et al* 1961) but further information regarding electrophysiological properties of the human fetal heart is lacking The aim of the present investigation is to provide data concerning resting membrane potential action potential characteristics with special reference to pacemaker activity and conduction velocity in various regions of the isolated human fetal heart at midgestation

Methods

Material Fetal hearts were obtained at legal interruptions of pregnancy performed by hysterotomy Over a period of 2 years, 24 heart preparations with no signs of malformations were used The gestational age ranged 17—24 weeks, the fetal crown — heel length was 7 5—25 cm.

Preparation of the hearts Preparation of the fetal heart was started immediately after the extraction of the fetus The dissection and mounting procedures were carried out at room temperature in an oxygenated Ringer s solution (see below) Three series of experiments were run In a first series consisting of seven isolated papillary muscle preparations the influence of external potassium concentration and temperature on resting membrane potential was studied The second series of hearts was used for recordings of action potentials from the sinus node the atria and the right ventricle and for conduction velocity measurements in atria and ventricles These preparations were dissected as follows The right ventricle was opened by an incision in the anterior wall near the interventricular septum. The anterior wall of the right atrium was cut near the inter atrial septum up to the superior caval vein antero-medially to the sinus node region The opened heart was placed in a thermostated bath Ringer s solution was flowed through the bath at a rate of approximately 2—3 ml/min. In the third series of experiments in which AV transmission was studied the hearts were perfused via the coronary arteries *ad modum* Langendorff with the preparation immersed in Ringer's solution in a thermostated bath For this purpose the right coronary artery had to be ligated after the *origin of the small branch to the sinus node area in order to* avoid shunting perfusion fluid directly to the bath The coronary perfusion pressure was approximately 30 cm of water and the flow rate 2—3 ml min In all experiments the hearts were fixed by means of small platinum needles to a bees-wax coated dish in the thermostated bath The temperature was maintained at 37 ± 0 5° unless otherwise stated.

Recording techniques For extracellular recording bipolar glass electrodes with Ag/AgCl wires in agar Ringer were used The tips of the electrodes were less than 0 5 *mm* in diameter For intracellular recordings of resting and action potentials an arrangement was used similar to that described by Niedergerke and Orkand (1966) The signals were fed into double-sided cathode followers and displayed on a Tectronix 502 A oscilloscope or an Elema Schonander Mingograph (*flat frequency response up to 500 Hz) The micro-electrodes* were boiled in methanol and afterwards placed in 3 M KCl for 2—5 days Micro-electrodes with resistances of 10—20 MΩ and tip potentials less than 20 mV were used except for determination of the resting membrane potential in ventricle cells when electrodes with tip potentials less than 5 mV were selected. The micro-electrodes were usually mounted directly to Prior micro-manipulators

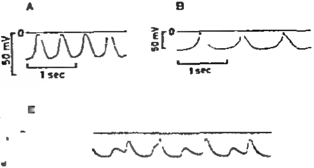


Fig 1 A B, C. Recordings from sinus node cells in three preparations Note the prepotential of

However, when studying transmission through the AV-node, it was found necessary to use 'floating' electrodes (Woodbury and Brady 1956). The micro-electrode insertions were car-

more than a few seconds

Solution A Ringer's solution of the following composition was used in all experiments, except when studying effects of changes in external potassium concentration (mM) NaCl 120, $NaHCO_3$ 25, KCl 4 0, $CaCl_2$ 2 0, NaH_2PO_4 1.5 $MgSO_4$ 1 5, dextrose 3 3 The pH of the

KCl in the ordinary Ringer's solution All chemicals were of analytical grade Glass distilled water was used for preparing solutions and for washing glass ware

Results

Action Potential Characteristics

1 The sinus node

In 6 preparations intracellular recordings from the lower part of the superior wall of the vena cava showed pacemaker potentials *i e* low resting membrane potentials, diastolic depolarizations, and slow upstrokes of the action potentials In two of these preparations steps or prepotentials on the upstrokes of the action potentials could be seen, in one heart simultaneous depolarizations from two independent foci were identified (Fig 1)

The area within which typical pacemaker potentials could be recorded from cells close under the endocardium did not exceed one mm² In most of the preparations, the transition between pacemaker and atrial types of action potentials was abrubt However recordings from atrial cells in particular those lying in the upper part of the atrium exhibited slow diastolic depolarizations (see below)

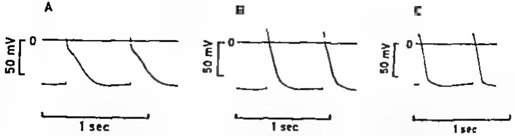
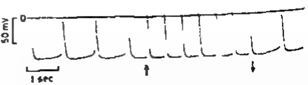


Fig. 2 A, B, C. Action potentials from atrial cells. A and B represent the most common variants; action potentials similar to that shown in C were occasionally seen.

2. The atrium

Action potentials from atria were recorded in eight preparations. Typical configurations of atrial action potentials are illustrated in Fig. 2 A, B. The rapid upstroke of the action potential was often followed by an incomplete plateau phase. The duration of the action potential varied within wide limits, in general being longer in the superior than in the inferior part of the atrium. Some atrial action potentials were of short duration and did not exhibit a plateau but showed an upwards convex curvature from the end of the upstroke until the later phase of repolarization (Fig. 2 C). Diastolic depolarizations were often seen in atrial cells, most frequently in the superior part of the atrium. These depolarizations could most easily be demonstrated at a low pacemaker frequency. This is illustrated in Fig. 3 by intracellular recordings from two atrial cells. When stimulated at a low frequency by impulses from the sinus node, the cells showed diastolic depolarizations. These depolarizations were masked, however, during rapid external stimulation when the diastolic interval is shortened. The action potentials were then triggered at more negative membrane potentials which increased the overshoots of the action potentials. Some atrial preparations in which the sinus node area had been removed were studied. Most of these preparations contracted spontaneously while some were quiescent. Intracellular recordings form cells in the quiescent preparations showed an interesting feature which might indicate latent pacemaker activity even in these preparations. At low external stimulation frequency, sub-threshold oscillations occurred (Fig. 4). The amplitude of these oscillations increased after a period of rapid external stimulation whereas their frequency remained approximately unchanged. It is unlikely that

Fig. 3. Recording from one atrial cell in the superior part of the atrium. The preparation is stimulated externally between the arrows. Note concealment of diastolic depolarizations and increase in amplitude of action potentials during external stimulation.



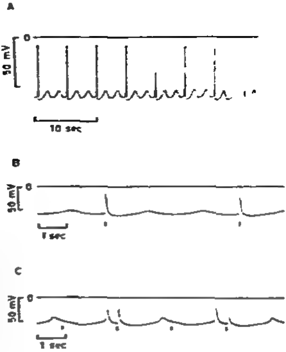


Fig. 4. Action potentials from one isolated atrial preparation without the sinus node. Vertical bars in B and C indicate stimulation. A and B stimulation frequency 12/min; the two curves are recordings from the same cell at different time scales. C stimulation frequency 30/min after stimulation at frequency 120/min. Note increase in amplitude of spontaneous oscillations and spike formation in response to every second oscillation.

these oscillations were due to mechanical damage of a particular cell, as a great number of cells were impaled all showing the same phenomenon.

3. *The AV-node*

Action potentials resembling those considered typical for the adult mammalian AV-node (Paes de Carvalho *et al.* 1959, Hoffman and Cranefield 1960) were found in the area beneath the opening of the coronary sinus. There was a gradual change between atrial and nodal types of action potentials. The transition of the lower part of the node into the bundle of His was difficult to study because of movements of the underlying ventricular muscle. In coronary perfused preparations the AV-nodes propagated impulses up to a frequency of 180—240/min. Fig. 5 shows action potentials obtained with floating electrodes from the superior and inferior part of the

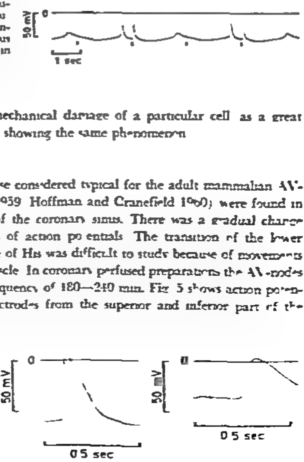


Fig. 5. Recordings from superior and inferior part of the AV-node from a coronary perfused preparation, upper and lower curve respectively. Stimulation artefact visible.

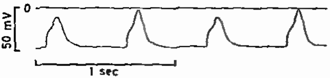


Fig 6 Action potentials from one AV node cell in a surface perfused preparation Note reduction in size of every second action potential and prominent notches in the beginning of the action potentials

node, corresponding to the AN and NH regions according to Paes de Carvalho and Almeida (1960) In the inferior part of the node, the magnitude of the resting membrane potential is small, and the velocity of upstroke of the action potential is low The distance between the two recording points in Fig 5 was 0 6 mm and the time interval between the two action potentials, measured at 50 % level of their rising phases was 170 msec Thus the mean conduction velocity under the experimental conditions (stimulation frequency 90/min temperature 35° C) was 0 0035 m/sec Further data concerning transmission through the AV node will be given in a following section (AV conduction)

In heart preparations immersed in Ringer s solution and perfused at their surfaces but without coronary perfusion conduction block regularly occurred within a short time after mounting When a transmission block could be localized it was found within or below the inferior part of the AV node In two such preparations recordings from the upper and middle part of the node were obtained Resting membrane potentials in cells from the middle of the AV node were about -50 mV and the action potentials showed deformations on their upstrokes During antegrade stimulation the time interval between this step or notch of the action potential and the following depolarization increased when the recording electrode was moved towards the inferior part of the AV node Fig 6 shows a recording from one cell in the middle of the AV node In addition to the prominent notch it should be noted that every second action potential is somewhat lower in amplitude than the preceding one although the resting membrane potential is virtually unchanged during the recording time This indicates an inactivation effect on the excitation mechanism without changes in resting membrane potential

4 The ventricle

At external potassium concentrations exceeding 1 mM the membrane potential remained stable during diastole in ventricle cells An action potential from a papil

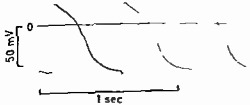


Fig 7 Action potentials from one cell in a papillary muscle preparation Stimulation frequency 120/min

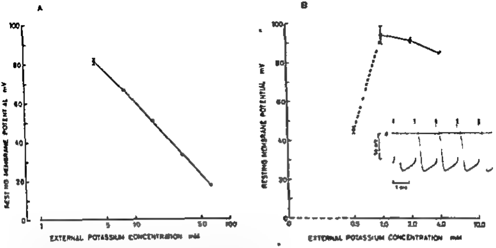


Fig 8 Resting membrane potential (inside negative) in fetal ventricle cells as a function of external potassium concentration, $(K^+)_o$.
6—12 impalements in each preparation at the concentrations indicated
A $(K^+)_o$=4—64 mM $(K^+)_o \times (Cl^-)_o$=480 mM²
Mean±SEM of five preparations
B $(K^+)_o$=0.5—4 mM Dotted line indicates region where spontaneous action potentials (see insert figure) appeared Mean±S D in one preparation

lary muscle preparation is illustrated in Fig 7 At a stimulation frequency of 120/min, the duration of the action potential, measured at 50 % level, was 230 msec

Resting membrane potential

The resting membrane potential in isolated nonbeating ventricle cells was studied in 5 preparations at external potassium concentrations varying from 4 to 64 mM In these experiments, the product of external potassium and chloride concentrations was kept constant by substitution of CH_3SO_4 for Cl^- mole per mole This was done in order to avoid changes in the internal potassium concentration by disturbing a Donnan equilibrium presumed to exist across the cell membrane As seen in Fig 8 A the resting membrane potential at an external potassium concentration of 4 mM was −81.4±1.6 mV At external potassium concentrations between 8 and 64 mM the resting membrane potential increased 55.2 mV per tenfold increase in external potassium concentration This is less than the 61.4 mV expected for a diffusion potential at 37° C determined solely by a potassium activity gradient In two experiments, one of which is shown in Fig 8 B the external potassium concentration was lowered in four steps from 4 to 0.5 mM The resting membrane potential was most negative in the solution containing 1 mM In 0.5 mM spontaneous action potentials with diastolic depolarizations occurred in ventricle cell before the resting membrane potential reached its final value −45.1 mV (see inset in Fig 8 B) Temperature dependence of the resting membrane potential was investigated in four preparations Within the range 19—37° C resting membrane potential became l

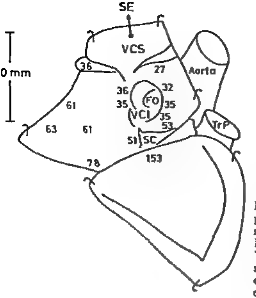


Fig 9 Schematic drawing of a fetal heart preparation showing spread of excitation within the right atrium SE stimulating electrode FO, foramen ovale VCS, vena cava superior VCI, vena cava inferior TrP, truncus pulmonalis SC, sinus coronarius Numbers indicate time in msec from stimulation to arrival of the impulse at the recording point

negative with decreasing temperature, the change being 0.9 (range 0.7—1.2) mV per °C

Resting membrane potentials in atrial cells as determined by six impalements in one spontaneously beating preparation (pacemaker frequency 108/min) were 71.2±4 mV (mean±S.D.)

Spread of excitation in atria

The spread of excitation within the right atrium was studied in two preparations one of which is shown in Fig 9 A stimulating platinum electrode was placed in the

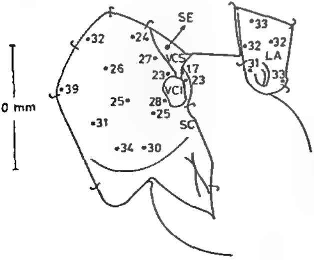


Fig 10 Schematic drawing showing spread of excitation in a fetal heart preparation in which muscle connexion between atria was saved Both atria opened LA left atrium other abbreviations and definition of numbers as in Fig 9

TABLE I Conduction velocity in human fetal atrium and ventricle at 36—37° C.

Preparation	Conduction velocity m/sec	Stimulation frequency min⁻¹	Temperature °C
RA	0.52	120	36
LA	0.36	140	36
LV	0.39	140	36
RA	0.48	140	36
LA	0.70	140	36
LV	0.38	140	36
RA	0.45	140	37
LV	0.48	120	37

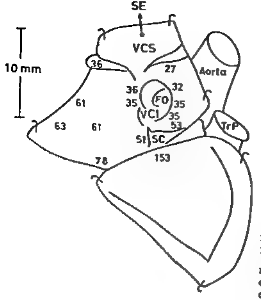
RA = right atrium
LA = left atrium
LV = left ventricle

TABLE II Temperature dependence for conduction velocity in human fetal atrium and ventricle

Preparation	Conduction velocity m/sec	Stimulation frequency min⁻¹	Temperature °C	Q_{10}
LA	0.27	100	27	1.5
	0.36	100	36	
LV	0.29	60	27	1.8
	0.46	60	36	
RA	0.37	100	27	2.1
	0.50	100	36	
LA	0.20	100	27	3.2
	0.60	100	36	
LV	0.21	60	27	2.2
	0.41	60	36	

RA = Right atrium
LA = Left atrium
LV = Left ventricle

sinus node region. The arrival of the impulse in different parts of the atrium was noted by means of extracellular bipolar electrodes. As can be seen the impulse reaches the upper border of the AV node in about 50 msec at a stimulation frequency of 120/min and temperature 37° C. Another 100 msec are required for the action potential to cross the node. (More data about conduction velocity in atrium, ventricle and AV node are given below.) Fig. 10 shows a schematic drawing of a preparation in which muscle connexion between the atria, including the bundle of Bachman, was saved as much as possible. It is evident that under the experimental



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negative with decreasing temperature, the change being 0 9 (range 0 7—1 2) mV per °C

Resting membrane potentials in atrial cells as determined by six impalements in one spontaneously-beating preparation (pacemaker frequency 108/min) were 71 2 ± 4 mV (mean ± S D)

Spread of excitation in atria

The spread of excitation within the right atrium was studied in two preparations, one of which is shown in Fig 9 A stimulating platinum electrode was placed in the

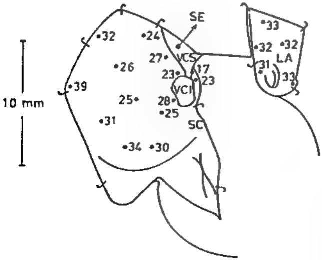
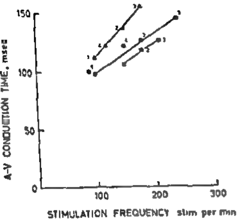


Fig 10 Schematic drawing showing spread of excitation in a fetal heart preparation in which muscle connexion between atria was saved Both atria opened LA left atrium, other abbreviations and definition of numbers as in Fig 9



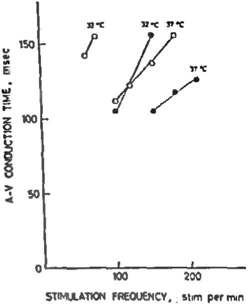


Fig 13 AV-conduction time in 2 preparations at temperatures 32° C and 37° C

AV-Conduction

The AV-nodal delay was investigated in coronary perfused preparations with two bipolar electrodes, one being placed lateral to the coronary sinus orifice, approximately 1 5 mm above and the other electrode 1 mm below the atrio-ventricular border at the interventricular septum The preparation was stimulated in the sinus node area The tissue between the two electrodes included, in addition to the AV-node, a small portion of adjacent atrial and ventricular myocardium However the time taken for the impulse to pass through the extranodal portions would not exceed a few percent of the total AV conduction time This estimate is based upon the presumption of a conduction velocity of 0 5 m/sec in atrium and ventricle (*cf* above)

Fig 12 shows the results of measurements of the AV-conduction time in 3 preparations The conduction time increased with increasing stimulation frequency up to 180—240 stimulations per min At higher frequencies conduction block occurred In two preparations influence of temperature on conduction time was studied (see Fig 13) At constant stimulation frequency, a decrease in temperature from 37 to 32° C caused approximately a 50 % increase in conduction time

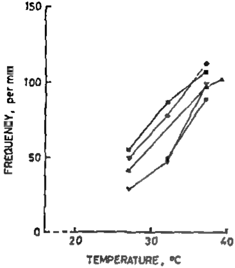


Fig 14 Spontaneous pacemaker frequency in 5 preparations at temperatures 27° C—37° C

Pacemaker frequency

The pacemaker frequency in the isolated, spontaneously beating, human fetal heart was 96 ± 66 beats per min at 37° C (M ± S E M from 12 preparations) The temperature dependence of the pacemaker frequency was studied in 6 preparations (Fig 14) As can be seen, the pacemaker frequency has a Q_{10}-value of approximately [illegible]

Discussion

Resting membrane potential in midterm fetal heart cells

When recording transmembrane potential differences across membranes of small cells such as myocardial cells, the possibility of leak currents around an inserted microelectrode should be taken into account Special caution must be taken when comparing data from cells of different size, such as adult and fetal heart cells (Couch *et al* 1969) Information concerning resting membrane potentials in adult human heart cells is scanty However, recordings from human atrial (Sleator and de Gubareff 1964) and papillary msucle preparations (Prasad and Callaghan 1969) show resting membrane potentials of the same amplitude as in midterm fetal heart cells The resting mebrane potential values obtained in the present study are also in accordance with those considered normal for adult mammalian heart cells (Page 1962, see also Hoffman and Cranefield 1960) Furthermore, the dependence of the resting membrane potential on the external potassium concentration is similar to that shown for adult mammalian ventricle cells (Page 1962). This seems to indicate that the internal potassium concentration in human fetal ventricle cells is of the same magnitude as in adult mammalian heart cells In this connextion, it is of interest that the internal potassium concentration has been found to be equal also in human midterm fetal and adult erythrocytes (Bengtsson *et al* in press).

Depolarization in low external potassium concentration as in the fetal ventricle cells in the present study has been observed in Purkinje fibres (Weidmann 1956) and interpreted as due to a decrease in potassium conductance (Noble 1965). Spontanous action potentials were seen in fetal ventricle cells at an external potassium concentration of 0.5 mM and in this respect these cells bear some resemblence to Purkinje fibres from adult mammalian hearts. Thus, increase in diastolic depolarization and appearance of spontaneous action potentials are regular findings in Purkinje fibres from sheep and calf heart (Vassalle 1965). A reduction of both external calcium and potassium concentrations however is necessary for the appearance of pacemaker activity in adult mammalian ventricle and atrial cells (Muller 1965).

Pacemaker activity

In human fetal heart at midterm the sino-atrial node has been found to surround almost the entire entrance of the superior vena cava into the right atrium (Robb *et al.* 1948). In the present investigation typical pacemaker potentials could only be demonstrated in a very limited area in the lower posterior part of the superior vena cava orifice. However recordings were only performed in superficial cells and thus the deeper parts of the nodal region were not explored. Morover a rapid pacemaker frequency in one part of the node might conceal spontaneous depolarizations in regions with lower diastolic depolarization rates. This would also tend to reduce the area from which typical pacemaker potentials could be recorded.

Intracellular recordings from the sinus node area often revealed a small abrupt step on the upstroke of the action potential (Fig. 1 B) markedly different from the slow diastolic depolarization of the pacemaker potential. It might be of the same type as the notch often observed in action potentials from cells in the AV-node (see below). In Fig. 1 C two types of depolarizations are shown. The responses are additive and occur with different frequencies. It is reasonable to suppose that one is the regular action potential, the other an electrotonic potential originating in neighbouring cells. Only the former was transmitted to the atrium. This indicates a local conduction block within the sinus node in accordance with the view that the safety factor of impul e propagation is low in this region (Hoffman and Cranefield 1960).

Diastolic depolarizations which did not cause excitation were generally found in cells in the upper part of the atria. They could most easily be seen when pacemaker frequency was low as in the recording shown in Fig. 3. The subthreshold oscillations found in two mechanically inactive preparations in the present study indicate that even seemingly quiescent preparations might have latent pacemaker properties. The depolarizing effects of such oscillations might explain the low conducti n velocity

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COHN, A, Physiological ontogeny A Chicken embryos VI Differentiation in the chicken embryo heart from the point of view of stimulus production *J exp Zool* 1925 *42* 299—310

COUCH, J R, T C WEST and H E HOFF, Development of the action potential of the prenatal rat heart *Circulat Res* 1969 *24* 19—31

DURRER, D, J BULLER, P GRAAF, G J LO and F L MEYLER, Epicardial excitation pattern as observed in the isolated revived and perfused fetal human heart *Circulat Res* 1961 *9* 29—38

FANGE, R, H PERSSON and S THESLEFF, Electrophysiologic and pharmacologic observations on trypsin — disintegrated embryonic chick hearts cultured in vitro *Acta physiol scand* 1956 *38* 173—183

GARREY, W E, E E TOWNSEND, Neural responses and reactions of the heart of a human embryo *Amer J Physiol* 1948 *152* 219—224

GENNSER, G, H W JOHANSSON and S KULLANDER, Electrocardiographic and tissue lactate changes in the hypoxic human fetus in mid pregnancy *J Obstet Gynaec Brit Cwlth* 1968 *75* 941—948

GENNSER, G and E NILSSON, The relation between the action potential and the active state in human fetal myocardium and its dependence on muscle length on contraction frequency *Acta physiol scand* 1968 *73* 42—53

HALL, E K, Further experiments on the intrinsic contractility of the embryonic rat heart *Anat Rec* 1954 *118* 175—184

HAUSWIRTH, O, D NOBLE and R W TSIEN, The mechanism of oscillatory activity at low membrane potentials in cardiac purkinje fibers *J Physiol* (Lond) 1969 *200* 255—265

HOFFMAN, B F and P F CRANEFIELD, *Electrophysiology of the heart* Mc Graw Hill Book Comp INC New York 1960

HOFFMAN, B F, A PAES de CARVALHO and W C DE MELLO, Transmembrane potentials of single fibers of the atrio-ventricular node *Nature* (Lond) 1958 *181* 66

LLOYD, W D M, The action of calcium on the isolated human foetal heart *J Pharmacol* 1929 *36* 185—193

MATSUDA K, T HOSHI and S KAMEYAMA, Action potential of the atrioventricular node *Tohoku J exp Med* 1958 *68* 8

MERIDETH, J, C MENDEZ, W MUELLER and G K MOE, Electrical excitability of atrioventricular nodal cells *Circulat Res* 1968 *23* 69—85

MULLER P, Ca- and K-free solution and pacemaker activity in mammalian myocardium *Helv physiol pharmacol Acta* 1965 *23* C 38—41

NIEDERGERKE, R and R K ORKAND, The dual effect of calcium on the action potential of the frog's heart *J Physiol* (Lond) 1966 *184* 291—311

NOBLE D, Electrical properties of cardiac muscle attributable to inward going (anomalous) rectification *J cell comp Physiol* 1965 *66* Suppl 2 127—136

PAES DE CARVALHO, A and D F DE ALMEIDA, Spread of activity through the atrioventricular node *Circulat Res* 1960 *8* 801—809

PAES DE CARVLHO A W C DE MELLO and B F HOFFMAN Electrophysiological evidence for specialized fiber types in rabbit atrium *Amer J Physiol* 1959 *196* 483—488

PAGE E Cat heart muscle *in vitro* II The steady state resting potential in quiescent papillary muscles *J gen Physiol* 1962 *46* 189—199

PRASAD K and J C CALLAGHAN Effect of replacement of potassium by rubidium on the transmembrane action potential and contractility of human papillary muscle *Circulat Res* 1969 *24* 157—166

ROBB, J S C T KAYLOR and W G TURMAN A study of specialized heart tissue at various stages of development of the human fetal heart *Amer J Med* 1948 *5* 324—336

SANO, T F SUZUKI and S TAKIGAWA, The genesis of the step or notch on the upstroke of the action potential obtained from the atrioventricular node *Jap J Physiol* 1964 *14* 659—668

SLEATOR, JR W and T DE GUBAREFF Transmembrane action potentials and contractions of human atrial muscle *Amer J Physiol* 1964 *206* 1000—1014

TRUEX, R C, J K BISHOP and E L HOFFMAN Accessory atrioventricular muscle bundles of [illegible] 1958 *131* 45—59

[illegible] and intracellular K concentra[illegible]

[illegible] Bern 1956

[illegible] moving tissues with a flexibl[illegible]

mounted ultramicroelectrode *Science* 1956 *123* 100—101

Acta physiol. scand. 1970. 79. 321—334

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Relation between Tension and Sensory Response of the Isolated Frog Muscle Spindle during Stretch

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Abstract

HUSMARK, I. and D. OTTOSON. *Relation between tension and sensory response of the isolated frog muscle spindle during stretch.* Acta physiol. scand. 1970. 79. 321—334.

The tension developed in the isolated frog muscle spindle during stretch has been studied and related to the impulse response and the isolated receptor potential of the spindle. With a steplike stretch tension rose to an initial peak and then declined towards a steady level. The decay consisted of an initial phase of rapid fall and a later phase of slow decline when stretch was prolonged. The magnitude of the peak and the static level of tension were exponential functions of the amount of stretch. The response of the spindle in terms of the impulse discharge and the isolated receptor potential closely followed the tension changes during various phases of stretch. Adaptation of the spindle to maintained stretch, in terms of the fall of the receptor potential, followed the same general time course as the decay in tension. The results suggest that tension during stretch reflects structural changes within the spindle and that these changes may bear a more direct relation to the sensory response than lengthening. Neither the gross tension nor the elongation can, however, be regarded as a direct representation of the actual stimulus to the endings.

The muscle spindle is a length measuring receptor which responds to passive stretch of the whole muscle or to active contraction of the intrafusal fibres. The mechanical stimulus is transmitted to the endings by the intrafusal fibres and the intracapsular connective tissue elements. It is plausible that in the course of this process the stimulus undergoes distortion. This would imply that the actual stimulus to which the endings are exposed is not identical to the external stimulus in terms of lengthening of the spindle. In studies of the isolated frog spindles which were subjected to controlled stretches it was found that the spindle response reproduced the properties of the mechanical stimulus with great precision except for the early period of maintained stretch (Shepherd and Ottoson 1965). In this period the frequency of the impulse discharge falls rapidly although stretch is maintained constant. With prolonged steady stretches the initial rapid fall in frequency is followed by a more gradual decline.

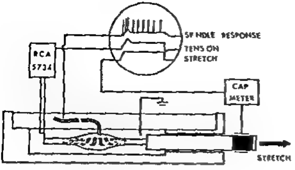

Fig. 1. Schematic diagram illustrating experimental set-up. Isolated spindle mounted in Ringer bath with sensory axon lifted up in oil. One end of spindle tied to balsawood lever fastened to anode of transducer (RCA 5734), the other end tied to fine nylon rod connected to coil of loudspeaker. Stretches monitored by capacitance meter measuring movement of fine metal rod attached to pulling nylon rod.

Recent studies of the crayfish stretch receptor (Krnjevic and Van Gelder 1961, Wendler 1963, Brown and Stein 1966) have focused interest on the relationship between the response characteristics of this receptor and the tension changes developed during stretch. It has been suggested (Krnjevic and Van Gelder 1961, Brown and Stein 1966) that tension actually may be the decisive stimulus.

The aim of the present study has been to study the tension changes produced by controlled linearly rising stretches of the muscle spindle. Particular attention was focused on the tension changes occurring during transition from dynamic to static stretch. It was found that the response of the spindle in terms of the impulse discharge and the isolated receptor potential closely followed the tension changes during various phases of stretch.

Methods

Preparation. Isolated muscle spindles of the frog's toe muscle (*m. ext. dig. long. IV*) were used in all experiments. The spindle was isolated by careful dissection and transferred to a small chamber containing Ringer's solution of the following composition: NaCl 110 mM, KCl 2.5 mM, $CaCl_2$ 1.8 mM, NaH_2PO_4 0.85 mM, Na_2HPO_4 2.15 mM, pH 7.1—7.4. In the chamber the spindle was tied at one end with a cotton wool filament to a fine nylon rod and at the other end to a vertical lever attached to the anode of a RCA 5734 transducer (Fig. 1). The length of the spindle was adjusted to take up slack until any further increase led to an increase in the spontaneous firing rate (*cf.* Ottoson 1961, Ottoson, McReynolds and Shepherd 1969). The temperature of the Ringer bath was kept at +18° C.

Stretch. The nylon rod to which the spindle was attached was fastened through a balsa wood lever to a loudspeaker coil (Philips 4 D 2300 BZ). Stretches were applied by driving the loudspeaker coil with electrical pulses of different waveforms. To avoid oscillations the loudspeaker was critically damped. With the fastest stretch used the 10—90 % rise time was about 3 msec.

In the present study linearly rising stretches at rates of about 120 mm/sec down to 0.5 mm/sec were used. The movement of the nylon rod was monitored on one oscilloscope beam by a high-sensitive capacitance meter (Haapanen 1962) which measured the movement of the lever as described earlier (Ottoson 1965). Since the nylon rod was relatively inelastic and rigidly fastened to the pulling lever the recorded displacement of the lever was assumed to represent the actual stretch applied to the spindle.

Tension recording. Tension was recorded with a RCA 5734 mechano-electric transducer mounted on a micromanipulator. To increase the sensitivity of the transducer a pin was attached to the anode. Various materials and shapes of the pin were tried in order to increase the gain of the transducer mechanically without lowering the resonant frequency of the recording system too much. A 20 mm long lever of balsa wood which in transverse section was rectangular (0.5 × 2 mm) was found to be the best compromise. At its end the lever carried an extension of a 1 mm long silver wire (diameter 100 μ) fastened to the lever with hard dental

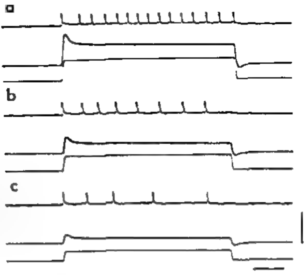

Fig 2 Impulse response and tension changes during maintained stretch of spindle to 25 per cent (*a*) 17 per cent (*b*) and 10 per cent (*c*) of resting length In these and following records the upper trace shows nerve response middle trace tension and lower trace extension Vertical bar 10 mg Time bar 40 msec

was The lever had a thin layer of lacquer to diminish absorption of oil or Ringer s solution which might have increased its weight The resonant frequency of the tension recording

rise time of stretch which was about 3 msec The delay of the system was determined by measuring the time lag for the onset of the return of the lever after release of the rod The delay was 0 5 msec It can be assumed that this is the time needed for the mechanical wave to travel through the lever to the anode of the transducer

The RCA transducer was coupled as one arm in a Wheatstone-bridge the other arms being high-quality wire wound low temperature-coefficient resistors A regulated power supply for heater and anode was used and the tube was kept at constant temperature by cooling the copper micromanipulator which held it

Under these circumstances the drift was minimal The RCA transducer was calibrated with the lever in a horizontal posit on by removing known weights from the lever and measuring the resulting deflections of the CRO beam The system was linear over the entire measuring range and the sensitivity of the bridge was 0 35 mV/mg The compliance of the system with the loads appl ed in the present study was negligible as judged from the fact that no displacement of the pin could be observed in the microscope even when force was twice the maximum applied

Recording of spindle response Recording of the response of the spindle was made with calomel half cell electrodes connected to the preparation through R nger agar bridges One electrode was placed in the bath while the other held the afferent nerve which was raised into paraffin o l The electrodes were connected by a cathode follower to a DC amplifier and the responses were displayed on one of the beams of the Tektronix oscilloscope The results from 20 sp ndles were analyzed in the present study

Results

Tension changes and impulse response

When the spindle was stretched in a steplike fashion the tension rose to a peak and then declined during steady stretch (Fig 2) The fall from the dynamic peak to the

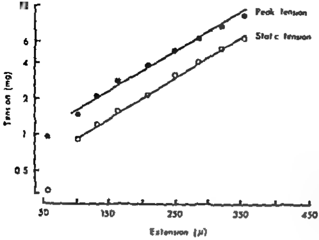

Fig. 3. Relationship between extension and tension. Filled circles dynamic peak of tension; open circles static tension at 150 msec after termination of dynamic stretch.

static level occurred in two phases: an initial rapid fall and a later phase of slow decline. At the termination of stretch the tension dropped transiently below the resting value. The time interval between the onset of the stretch and that of increase in tension was of the order of 0.5 msec as measured from the oscillographic recordings. Since the time lag of the tension recording system was about 0.5 msec (see Methods) it would appear that there was no appreciable delay involved in the onset of the increase in tension. This in turn may be explained by the short length of the spindle (usually about 1 mm): tension thus developed with negligible delay throughout the entire spindle when stretch was applied.

At threshold the tension values varied a great deal from one preparation to another. This was most likely due to differences in resting tension. As described in Methods the resting tension was determined by stretching the spindle until further lengthening produced an increase in the spontaneous discharge (cf. Ottoson et al. 1969). Since the length at which the spontaneous discharge began to increase could not be determined exactly, differences in resting tension were bound to occur in different spindles. The mean tension developed at threshold in four experiments was 0.5 mg with a range of 0.2—0.8 mg. The maximum dynamic receptor potential in response to a fast rising stretch is usually obtained by extending the spindle by about 30 % of its resting length (Ottoson and Shepherd 1965). The tension produced with such a stretch was about 5—12 mg. This would imply that the working range of the spindle from threshold to the maximum dynamic response to a sudden stretch extends from a few tenths of a mg to about 10 mg.

The quantitative relationship between the amount of stretch and the tension developed with step stretches to different lengths is illustrated in Fig. 3. As can be seen the peak dynamic tension for a given velocity of stretch is an exponential function of the amount of lengthening of the spindle. The static values have also been included in the diagram for comparison. The two lines are approximately parallel indicating that static tension is a constant fraction of peak tension.

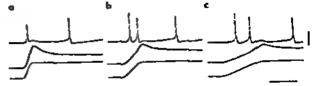

Fig 4 Records illustrating changes in tension with different velocities of extension Vertical bar [illegible] mg Time bar 10 msec

When stretch is applied rapidly tension during dynamic stretching reaches higher values than with more slowly rising stretch as illustrated in Fig 4 Plotting of the data for peak tension and lengthening on the spindle at different velocities shows (Fig 5) *that for a given velocity of stretch the values tend to fall on a nearly straight* line and that the lines for different velocities are approximately parallel With increasing velocity the length tension diagrams are successively higher indicating that the peak tension is a regular function of the velocity of stretch

The impulse response of the spindle to stretch is characterized by an initial dynamic discharge followed by a decline in frequency to a steady level during sustained stretch It has been demonstrated earlier (Shepherd and Ottoson 1965) that for a given velocity of stretch the impulse frequency increases linearly with lengthening of the spindle A corresponding analysis of the tension frequency relationship is made in the diagram in Fig 6 The diagram illustrates the interrelationship between dynamic impulse frequency velocity of stretch and tension As can be seen for a given velocity of stretch the dynamic frequency increases linearly with the logarithm of tension In this particular diagram the velocities range between 16 and 2 8 mm/sec Within this range the slopes of the individual frequency tension curves appear to be parallel Measurements with faster stretches however showed that the slopes became increasingly steeper and the opposite was true for slower stretches

In the early period of maintained weak or moderate stretch tension falls from its dynamic peak towards a level from which it declines more slowly for the duration of steady extension An analysis of the time characteristics of the initial phase of decay

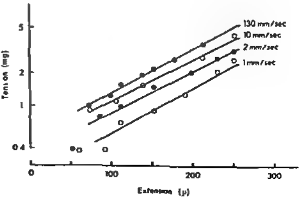

Fig 5 Relationship between dynamic peak of tension and extension at different velocities of stretch Spindle length 1 mm Semilogarithmic scales

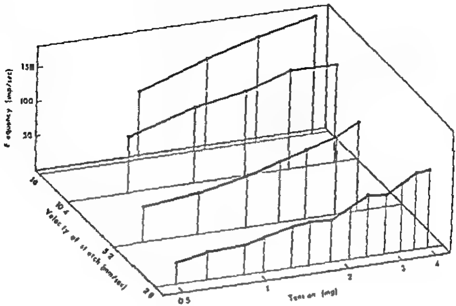

Fig. 6. Relationship between dynamic impulse frequency and tension for stretches at four velocities. Frequency measured as reciprocal of interval between spikes. Right horizontal axis: tension (logarithmic scale). Left horizontal axis: velocity of stretch (logarithmic scale). Vertical axis: impulse frequency (linear scale).

shows that tension falls exponentially with a time constant of about 5—12 msec. Within a given range of changes in amount of stretch the time constant of the fall remains relatively unchanged. With decreasing velocities of stretch to the same length the time constant of the fall in tension also remains unchanged or increases slightly.

A detailed study of the relation between the time characteristics of the decay in tension and the changes in firing frequency is limited by the fact that the impulse frequency is low with weak stretch while with stronger stretches a firing pause appears (Shepherd and Ottoson 1965). To relate the change in impulse discharge with that of tension the *dynamic index* (Crowe and Matthews 1964), i.e. the difference between the dynamic peak frequency and the static frequency, was plotted against the difference between dynamic peak tension and static tension. Since in the present study comparatively short stretches were used the static frequency and the tension were measured 150 msec after the termination of dynamic stretch. The data obtained from recordings with different amplitudes and velocities of stretch showed that changes in static tension always were accompanied by corresponding changes in impulse frequency. A closer analysis of the exact relationship between tension and impulse frequency was not possible because of the small range of changes of the two parameters.

In all experiments reported in the present study stretch was released by the re

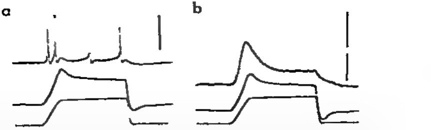

Fig 7 Isolation of receptor potential *a* tension and impulse response to a brief static stretch *b* tension and receptor potential to same stretch after blocking of conducted activity with 0 18 per cent lignocaine Upper vertical bar in *a* 2 mV, in *b* 1 mV Lower vertical bar 3 mg Time bars 20 msec

turn of the pulling rod within 2—3 msec to its original zero position Following termination of stretch tension fell abruptly Usually there was an undershoot followed by a gradual return to the resting tension level Concomitant with the fall in tension the discharge of the spindle ceased and there was a silent period before the spontaneous firing reappeared

Tension changes and receptor potential

In recordings of the composite response of the spindle the features of the receptor potential are usually not readily determined The receptor potential may however be obtained in isolation by treating the spindle with local anesthetics as was first shown by Katz (1950) In the present study lignocaine at a concentration of 0 18 % was used to block the conducted activity With this concentration all signs of conducted activity were abolished while the receptor potential appeared to be unaffected and remained stable for many hours The records in Fig 7 illustrate the features of the response before and after blocking and of the simultaneously recorded tension changes The spindle in this experiment was subjected to a relatively brief and weak

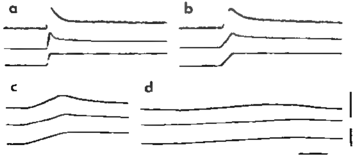

Fig 8 Changes of dynamic phase of receptor potential and tension with decreasing velocity of stretch Vertical bars upper 2 mV lower 5 mg Time bar 40 msec

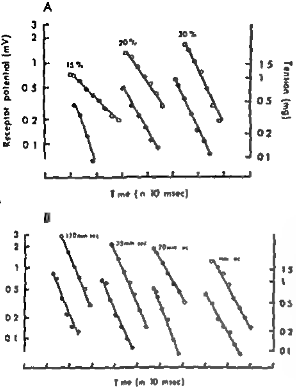

Fig. 9. Decay of receptor potential and tension from peak to static level for different amounts of stretch at same velocity (*A*) and for different velocities of stretch to same level (*B*). Open circles: receptor potential. Filled circles: tension. In each diagram left vertical axis gives amplitude of receptor potential and right vertical axis amplitude of tension. Time in 10 msec. Semilogarithmic scales.

stretch which produced two dynamic spikes followed by two static ones. The middle trace shows the tension produced by the stretch. Record *b* was obtained after blocking the conducted activity. In order to bring out the typical features of the receptor potential the gain of the recording from the nerve was increased. There is a striking similarity between the tension changes and the receptor potential. It may be noted, however, that the receptor response is not identical in shape with the tension changes. Thus the initial fall of the receptor potential is relatively greater than the corresponding fall in tension. Furthermore after termination of stretch tension drops abruptly while the receptor potential returns gradually towards the baseline.

For a given amount of stretch the magnitude and rate of rise of the receptor potential are functions of the velocity of stretch as described earlier (Ottoson and Shepherd 1965). Tension varies in a similar way as is illustrated in Fig. 8. With the fast rising stretch in *a* both the receptor potential and the tension rose rapidly to a peak and then declined to the static level. As stretch was applied more slowly (*b*–*d*) the rate of rise of the response and that of tension decreased and their dynamic amplitudes became lower while the static levels remained unchanged.

Following transition from dynamic to static stretch, tension falls at first rapidly and then more gradually when stretch is prolonged as described above. The receptor potential exhibits closely similar properties. In Fig. 9 A the initial rapid decays of the

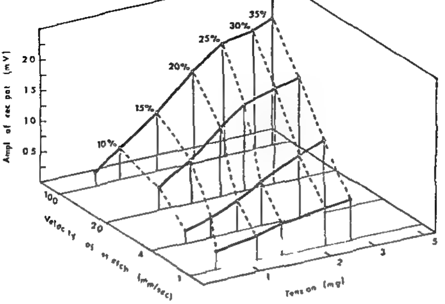

Fig 10 Relationship between amplitude of dynamic peak of receptor potential and tension for dynamic stretches at four velocities to same final level Right horizontal axis tension

receptor potential elicited by stretching the spindle with constant velocity to different lengths are plotted versus time, together with the corresponding values for tension The diagram shows that both tension and the receptor potential decayed exponentially For the strong stretch (30 %) the decay of tension is nearly parallel with that of the receptor potential while for the weak stretch (15 %) the two lines have different slopes It should be pointed out that the difference in time course of the decays for different velocities of stretch varied from one spindle to another In some experiments the changes of the receptor potential and those of tension were more or less equal so that for a given stretch the two decays had similar time constants In general, however the alterations in the decay of the receptor potential with changes in amount of stretch were more pronounced than those in the fall in tension In contrast to the changes occurring when the amount of stretch was varied the time constants of the decays remained relatively unaffected when the velocity of stretch was altered The diagram in Fig 9 *B* shows the decay curves for stretches at four velocities to the same final length The data are derived from the same spindle as in Fig 9 *A* The stretch was slightly below the strength required to give the maximum dynamic receptor potential As can be seen for any given velocity of stretch the receptor potential and the tension fell with approximately the same time constant

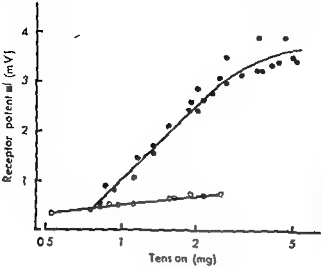

Fig 11 Relationship between *receptor potential* and *tension* Filled circles dynamic peak of receptor potential plotted against that of tension Open circles static receptor potential 50 msec after onset of sustained stretch plotted against tension at same time Semilogarithmic scales

The decays for different velocities were also similar and together the curves form a family of parallel lines

The graph in Fig 10 gives a *three dimensional schematic representation* of the interrelationship between length tension, velocity of stretch and the dynamic receptor potential In this experiment the spindle was stretched at four velocities ranging from 1 to 100 mm/sec up to 30 % of its resting length The data obtained may be assumed to cover the greater part of the range of variations in the parameters of the stimulus to which the spindle would be exposed under physiological conditions The increase of the potential with increasing stretch is represented by the heavy continuous lines The dotted lines indicate values for stretching the spindle at *different velocities* to the same *final length* *If attention is focused* on the heavy lines it will be noted that for a given velocity of stretch the amount of dynamic depolarization within the greater part of the physiological range is a linear function of the logarithm of tension The dotted lines show that for a given amount of stretch the dynamic depolarization increases linearly with the logarithm of velocity of stretch (*cf* Ottoson and Shepherd 1965) The relationship between the three parameters as illustrated in Fig 10 was a regular finding in all experiments although the absolute values varied from one spindle to another

As illustrated in Fig 11 the static level of the receptor potential also increased linearly with the logarithm of tension However the absolute values for static tension are considerably lower than the peak dynamic ones The peak dynamic tension produced by the strongest stretch used in the experiment illustrated in Fig 11 was about 5 mg This stretch was just strong enough to produce the maximum dynamic response of the spindle The static tension developed with the same stretch was only about 3 5 mg The maximum level of static depolarization of the endings was not reached at this amount of tension In order to obtain the maximum static response

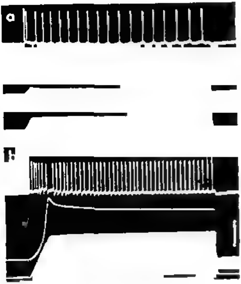

Fig 12

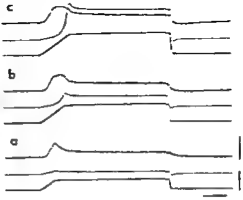

Fig 13

Fig 12 Tension changes and impulse response with overstretch Records obtained by stretching spindle to 16 per cent (*a*) and 60 per cent (*b*) of resting length. Vertical bar 20 mg Time bar 50 msec

Fig 13 Overstretch. Receptor potential and tension during extension of spindle to 25 per cent (*a*) 45 per cent (*b*) and 55 per cent (*c*) of resting length Vertical bars upper 2 mV lower 20 mg Time bar 40 msec

the spindle usually had to be stretched by about 100 % of its resting length (*cf* Ottoson and Shepherd 1965) This implies a considerable overstretch and was therefore avoided in most of the experiments in the present study

Overstretch

As shown by Shepherd and Ottoson (1965) the maximum dynamic discharge of the spindle is obtained at an extension of about 30 % of the resting length when stretch is applied rapidly For this reason great care was taken not to extend the spindle beyond this value and not to subject the spindle to prolonged stretches However in a few experiments the effect of overstretch was also studied The records in Fig 12 show the results obtained in one of these experiments A comparatively slow stretch was chosen in this experiment In *a* the spindle was subjected to a moderately strong stretch which gave rise to two dynamic spikes followed by a train of impulses during the ensuing phase of maintained stretch The tension developed during dynamic stretching appears to rise linearly at this strength of stretch and with the low gain used for the tension recording In *b* the spindle was extended by 60 % Inspection of the record shows that the impulse frequency increases for the first four spikes and then decreased toward the end of the period of dynamic overstretch The records also bring out the typical change in tension. As extension proceeds tension

rises successively faster *There is thus a difference in behaviour between the development* of tension and the impulse response When the spindle is stretched beyond what may be considered as the physiological range the dynamic frequency remains constant or decreases while tension continues to rise

For comparison the development of the receptor potential is illustrated by the records in Fig 13 In this experiment the spindle was subjected to a relatively slow stretch to bring out the differences between the receptor potential and the changes in tension more clearly In record *a* stretch was just strong enough to give the maximum dynamic phase of the receptor potential With stronger stretch (*b* and *c*) a plateau phase developed For a given velocity of stretch the amplitude of this dynamic plateau appears to represent the maximum depolarization of the sensory nerve endings It also indicates the upper limit of the physiological range of stretch For stretches below this level there is a close correlation between dynamic receptor potential and tension as described above When stretch goes beyond the physiological range this relation does not hold Tension then increases progressively while the depolarization of the sensory nerve endings remains relatively unchanged

Discussion

In the present study tension has been measured in isolated frog spindles which were subjected to controlled linearly rising stretches The preparation used seems particularly suitable for a study of this kind since it consists virtually only of the spindle itself with the intracapsular portions of the intrafusal fibres together with the nerve fibre and its sensory endings The tension changes recorded at one end of the spindle when controlled stretches are applied at the other end may therefore be considered to reflect relatively closely the tension to which the endings are exposed The results suggest that the overall mechanical properties of the muscle spindle are similar to those of the extrafusal muscle fibre Thus tension is an exponential function of lengthening in the spindle as well as in whole muscle (Hill 1949 Matthews 1959) or in bundles of isolated frog muscle fibres (Buchthal 1942) Since there is a linear correlation between firing rate and lengthening (Shepherd and Ottoson 1965) it follows that the response of the spindle in terms of discharge frequency is a logarithmic function of the tension as illustrated in Fig 3 and 5 This is in agreement with the observations by Matthews (1931) on non isolated frog spindles It is of interest to note that this relationship holds true for both the dynamic and the static response Terzuolo and Washizu (1962) reported a similar relationship between static firing frequency and length of the crustacean stretch receptor while Krnjevic and Van Gelder (1961 and Wendler (1963) found a linear or nearly linear relationship between the dynamic as well as the static discharge and tension These discrepancies might partly be explained by differences in the technique applied for stretching the receptor Krnjevic and Van Gelder (1961 used a manually operated micrometer for stretching while Wendler (1963) employed an electromagnet In both these studies the velocity of stretch was slow Furthermore Krnjevic and Van

Gelder (1961) as well as Wendler (1963) used prolonged superimposed stretches As pointed out by Brown and Stein (1966) this procedure may lead to overstretching of the receptor and produce changes in the relationship between length and tension Brown and Stein (1966) using linearly rising stretches of different velocities found linear correlations between static impulse frequency and both muscle length and tension However, the correlation was slightly higher between frequency and tension than between frequency and length and they therefore concluded that tension rather than muscle length represented the decisive stimulus to the stretch receptor

The present results show that with respect to some of the features of the dynamic and the static discharge the response of the muscle spindle is equally closely related to tension as to length, the impulse frequency being a linear function of length and a linear function of the logarithm of tension. However, the adaptive decline in firing during steady stretch can not be related to length changes in terms of alternations in the external length of the spindle while on the other hand the response characteristics during this period are similar to the tension changes The decay of tension during maintained steady stretch is of particular interest in relation to the problem of the mechanisms underlying the adaptation of the spindle As shown by Katz (1961) there is a marked structural difference between the central reticular region of the spindle and the polar regions This difference is undoubtedly important for the function of the spindle but the relationship between the response characteristics and the structural properties of the different regions of the spindle is not yet understood Photomicroscopical studies of isolated spindles during dynamic and static stretch have failed to reveal any gross length changes within the spindle (Ottoson and Shepherd 1968) It is most likely therefore that length changes if they occur, take place at the ultrastructural level The fact that there is a rapid fall in tension provides indirect evidence that a structural rearrangement takes place within the spindle in the early period of maintained stretch It would appear therefore that tension changes reveal mechanical alterations within the spindle which are not reflected in gross length changes In many of the experiments in the present study the time constant of fall in tension in the early period of maintained stretch was the same as that for the decay of the receptor potential and changes in amplitude or velocity of the stretch were often accompanied by equal changes in the time characteristics of the decline of the receptor potential and that of tension.

The observed differences between tension and the receptor potential with respect to the time course and magnitude of the fall from the dynamic peak to the static level might be partly explained by the fact that the spindle is not structurally homogeneous The complexity of the structural organization of the spindle together with the differences in visco-elastic properties between the central and the polar zones suggest that all the sensory nerve endings are not affected to the same extent during stretch Such differences would not be revealed by external measurements of overall tension while on the other hand they would be reflected in the recorded receptor potential

The present observations show that in certain respects the response of the spindle is more closely related to tension than to elongation This would appear to support the conclusion that tension may be regarded as a representation of the actual stimulus to the sensory terminals as has been suggested for the crustacean stretch receptor (Krnjevic and Van Gelder 1961, Brown and Stein 1966) However, it is a plausible assumption that the decisive excitatory factor is the deformation of the sensory membrane and that therefore neither lengthening nor tension can be considered to represent the structural alterations in the sensory membrane Nevertheless as an indirect representation tension may be a more adequate measure of the stimulus than lengthening since tension appears to reflect changes within the spindle which are not revealed by gross length changes

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References

Buchthal F The mechanical properties of the single striated muscle fibre at rest and during contraction and their structural interpretation *Dan Biol Medd* 1942 *17*, no 2

Brown M C and R B Stein Quantitative studies on the slowly adapting stretch receptor of crayfish *Kybernetik* 1966 *3* 175–185

Crowe A and P B C Matthews The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles *J Physiol Lond* 1964 *174* 109 131

Haapanen L E A high-sensitivity capacitance meter *Electronic Eng* 1962 *34* 183–185

Hill A V Is relaxation an active process? *Proc roy Soc B* 1949 *136* 420–435

Katz B Depolarization of sensory terminals and the initiation of impulses in the muscle spindle *J Physiol Lond* 1950 *111* 261 282

Katz B The termination of the afferent nerve fibre in the muscle spindle of the frog *Phil Trans B* 1961 *243* 221 240

Krnjevic K and N M van Gelder Tension changes in crayfish stretch receptor *J Physiol Lond* 1961 *159* 310 325

Matthews B H C The response of a single end organ *J Physiol (Lond)* 1931 *71* 64–110

Matthews P B C The dependence of tension upon extension in the stretch reflex of the soleus muscle of the decerebrate cat *J Physiol Lond* 1959 *147* 521 546

Ottoson D The effect of acetylcholine and related substances on the isolated muscle spindle *Acta physiol scand* 1961 *53* 276 287

Ottoson D The effects of temperature on the isolated muscle spindle *J Physiol (Lond)* 1965 *180* 636 648

Ottoson D J S M Reynolds and G M Shepherd Sensitivity of isolated frog muscle spindle during and after stretching *J Neurophysiol* 1969 *32* 24 34

Ottoson D and G M Shepherd Receptor potentials and impulse generation in the isolated spindle during controlled extension *Cold Spr Harb Symp quant Biol* 1965 *30* 105–114

Ottoson D and G M Shepherd Changes of length within the frog muscle spindle during stretch as shown by stroboscopic photomicroscopy *Nature Lond* 1968 *220* 912 914

Shepherd G M and D Ottoson Response of the isolated muscle spindle to different rates of stretching *Cold Spr Harb Symp quant Biol* 1965 *30* 95 103

Terzuolo C A and Y Washizu Relation between stimulus strength generator potential and impulse frequency in stretch receptor of crustacea *J Neurophysiol* 1962 *25* 56–66

Wendler L Über die Wirkungskette zwischen Reiz und Erregung *Z vergl Physiol* 1963 *47* 279–315

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Properties of Muscle and Liver Lysosomes in Adrenalectomized Rats

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Abstract

BERG T and J W C BIRD *Properties of muscle and liver lysosomes in adrenalectomized rats* Acta physiol scand 1970 79 335—350

The stability of lysosomes from liver and muscle of adrenalectomized young rats has been tested by measuring the release of acid hydrolases (RNase, cathepsin D β glucuronidase and aryl sulphatase) from lysosomes under various conditions Adrenalectomy increased the proportion of enzyme released from the
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stability of muscle and liver lysosomes *in vitro* was tested by exposing particles to osmotic stress vitamin A alcohol and acid medium (pH 5) Liver and muscle lysosomes from adrenalectomized rats were less stable when exposed to osmotic stress or vitamin A but hepatic lysosomes were more stable towards acid incubation than particles prepared from normal rats Differential centrifugation of liver homogenates indicated that more acid hydrolases were associated with the heavier fractions in homogenates from operated animals An increased particle size in tissues from the adrenalectomized animals may possibly account for the observed changes in lysosomal stability

Considerable attention has been paid to release of lysosomal enzymes because of the injurious effects they may cause Lysosomal rupture has been associated with pathological states involving certain shock conditions (Janoff *et al* 1962, Kovanic 1968) hypervitaminosis A (Weissman 1964) inflammation (Janoff and Zweifach 1964) and ischemia (de Duve and Beaufay 1959) De Duve postulated (De Duve *et al* 1962) that mechanisms must exist whereby the release of potentially injurious acid hydrolases may be facilitated or retarded and subsequently several biological agents were found that stabilized or labilized the lysosomal membrane, *i e* prevented or facilitated the release of enzymes from the particles Chief among the stabilizers are the anti inflammatory corticosteroids (Weissmann 1965) Glucocorticoids have, for

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instance, been shown to protect rabbit hepatic lysosomes against vitamin A and streptolysins both *in vitro* and *in vivo* (Weissmann and Thomas 1963, Weissmann *et al.* 1964). Cortisol and cortisone have also been shown to be effective in retarding the release of hydrolases from rat liver lysosomes exposed to low pH (de Duve *et al.* 1962).

Adrenal insuffiency leads to increased sensitivity to several stress situations which have been associated with increased release of lysosomal enzymes. These include traumatic shock, endotoxemia, infections and starvation. We considered it possible that the decreased resistance towards stress conditions observed in the adrenalectomized rat may be related to an abnormal release of acid hydrolases. The purpose of the present investigation was to evaluate the stability of liver and muscle lysosomes from adrenalectomized rats towards some treatments that are known to enhance the release of enzymes from hepatic lysosomes. *In vitro* release of enzymes were measured following exposure of the particles to (1) low pH, (2) vitamin A alcohol, and (3) osmotic stress. To study a possible *in vivo* enzyme release or increased mechanical fragility of lysosomes, the relative proportions of enzyme remaining unsedimentable after high speed centrifugation of tissue homogenates were measured directly. The enzymes chosen for this study were cathepsin D, arylsulfatase, β-glucuronidase and "acid ribonuclease". The location of these enzymes in hepatic lysosomes has been established (Straus 1967). Previous reports from this laboratory (Bird *et al.* 1968, Pollack and Bird 1968) have demonstrated that these enzymes are lysosomal also in muscle tissue.

Starvation may lead to *in vivo* release of lysosomal enzymes (Beaufay *et al.* 1959) or formation of fragile autophagic vacuoles (Swift and Hruban 1964). Since the adrenalectomized rat has a subnormal food intake, possible starvation effects were also investigated by studying normal animals pair-fed to the adrenalectomized animals.

Material and Methods

A. Animals and diet

Weanling male Sprague-Dawley rats were maintained on Purina Fox Chow until they attained a body weight of 60—65 g, at which time they were put on experiment. A semipurified 20 per cent casein diet (Leathem and Wolf 1954) was fed to all animals on experiment. In one series of experiments untreated adrenalectomized animals and their controls were sacrificed 5 days post adrenalectomy. Both pair-fed and *ad libitum* fed intact animals served as controls. In another series of experiments adrenalectomized animals were given 1 per cent saline as drinking water and sacrificed with their pair-fed controls 10 days after operation. In replacement therapy studies, a suspension of corticosterone in 50 per cent (v/v) menstruum (Squibb) was injected subcutaneously. Adrenalectomized animals were given either a threshold dose (300 μg) or a high dose (3 mg) of hormone daily for 5 days.

B. Operation

Adrenal glands were removed under light ether anesthesia through bilateral incisions in the lumbar region (Ingle and Griffith 1942). Care was taken to remove the glands with as much adherent fat as possible.

C. Preparation of homogenate

Animals were sacrificed by decapitation with a minimum amount of prior handling. Liver and gastrocnemius muscles were rapidly excised and chilled in ice-cold 0.25 M sucrose solution

homogenizer was kept in an ice slurry during the entire homogenization procedure Both tissues were homogenized in approximately 4 volumes of sucrose/EDTA solutions. The muscle homogenate was filtered through a layer of cheesecloth and both muscle and liver were finally diluted to 10 per cent (w/v) suspensions by sucrose/EDTA

D *Enzyme assays*

Cathepsin D was determined according to the method of Anson (1938) as modified by Bird *et al* (1968) Acid denaturated Bovine hemoglobin (1 67 per cent w/v) was used as a substrate and the pH of the incubation mixture was kept at 3 8 by 0 27 M acetate buffer The procedure used for the assay of acid ribonuclease was based on the method of de Duve *et al* (1955) Yeast RNA (0 033 per cent w/v) served as substrate and 0 08 M acetate buffer was used to keep the incubation mixture at pH 5 Aryl sulphatase (Roy 1953) was assayed in

enzyme activities were linear with time and enzyme concentration under the assay conditions used Muscle preparations were in most cases incubated for one hour Kinetic studies done in this laboratory with ribonuclease cathepsin D and arylsulphatase have demonstrated that under the assay conditions used the reactions are linear with time and enzyme concentration (Berg 1968 Bird *et al* 1968) In preliminary experiments on muscle β glucuronidase the effects of pH, enzyme concentration substrate concentration and time on β glucuronidase activity were studied A 10 per cent (w/v) muscle homogenate treated with 0 2 per cent (v/v) Triton X 100 (Rohm and Haas, Philadelphia Pa was used

The enzyme was found to be saturated with substrate at about 0 4 mM substrate concentration with the apparent k_m of about 0 125 mM It is also found that the enzyme activity is linear with enzyme concentration and time of incubation for at least 6 hrs In studies of the effect of pH on β glucuronidase activity acetate buffer was used to vary the pH of the incubation mixture between 4 0 and 6 0 A broad peak of activity appeared around pH 4 5

Protein was determined by the method of Lowry *et al* (1951) with Bovine albumen (Sigma) as the standard

All substrated used in enzyme assays were purchased from Sigma Company (St Louis Missouri)

E *Tissue Fractionation*

The amount of enzyme released from the lysosomal particles in fresh untreated tissue homogenates was measured in the supernatant after centrifugation of the homogenate in a Spinco Model L Ultracentrifuge at $78\,000 \times g$ for 45 min The amount of enzyme remaining in the lysosomes was measured by rupturing the particles with detergent (Triton X 100 either directly in the homogenate or in the sedimented particle fractions

A more complete fractionation procedure was used in some experiments on liver homogenates A nuclear fraction was separated at $1000 \times g$ for 7 min and the remaining cytoplasmic extract was devided into a heavy mitochondrial fraction ($10\,000 \times g$ for 5 min a light mitochondrial fraction ($22\,000 \times g$ for 10 min a microsomal fraction ($78\,000 \times g$ for *45 min) and a soluble fraction (final supernatant) All fractions except the final supernatant* were treated with Triton X 100 (0 2 per cent v/v

F *In Vitro Studies*

For studies of *in vitro* release of hydrolases from muscle and liver lysosomes a lysosome rich fraction was prepared between $1000 \times g$ for 7 min and $22\,000 \times g$ for 15 min The resulting pellet was washed twice and resuspended to the desired volume by a gentle up and down

TABLE I Food intake and body weight changes

Animal group	Number of animals	Initial body weight (g)	Body weight change (g)	Food intake (g)
Adx untreated	33	63 5	0 3±1 6	5 2
Pair fed control	16	62 8	3 7±1 7	5 2
Ad lib fed control	20	62 1	20 7±2 1	8 0
Adx saline treated	19	62 8	24 0±2 2	6 4
Pair fed control	*19*	*62 0*	*30 8±2 4*	*6 4*

Values represent means and where indicated ± standard errors of means Adx = adrenalectomized

for β glucuronidase and was done by incubating particle suspensions in presence of 0 05 M acetate buffer, pH 5 and 1 mM phenolphthalein glucuronic acid 0 25 M sucrose was included in the medium to provide osmotic protection to the lysosomes and the incubation time was kept short (10 min) to avoid excessive release of enzyme as a result of the low pH

In order to measure the *in vitro* release of enzyme from lysosomes exposed to low pH the particles were incubated at 37° C in 0 25 M sucrose and 0 05 M acetate buffer pH 5 At different time intervals aliquots of particle suspensions were removed from the incubator and either free or unsedimentable enzyme activities were measured Osmotic fragility of lysosomes was tested by the method of Gianetto and de Duve (1955) Lysosomerich fractions were mixed with equal volumes of sucrose solutions of different concentrations so that the final sucrose concentrations in the incubation mixture were 0 05 0 15 0 25 or 0 45 M These suspensions were incubated for 30 min at 0° C and then brought back to 0 25 M sucrose by adding a sucrose solution of appropriate concentration The final suspensions were assayed for free β glucuronidase activity The effect of vitamin A alcohol on the integrity of lysosomes was tested by incubating liver and muscle fractions in a neutral sucrose (0 25 M) solution containing 5×10^{-4} M vitamin A The vitamin was dissolved in 95 percent ethanol and added to the incubation mixture in 0 1 M concentrations Fractions were incubated under nitrogen and at 0 30 and 60 min 5 ml aliquots were removed from the incubator and recentrifuged Un sedimentable β glucuronidase activity was used as an indicator of *in vitro* release of lysosomal enzymes

Results

A *Food intake and Growth*

Food intake and body weight changes for untreated and saline treated adrenalectomized rats and control animals are shown in Table I The data indicate that the untreated adrenalectomized animal does not gain in weight and has a subnormal food intake That the inability to gain weight is due to the subnormal food intake is indicated by the fact that intact pair fed animals show comparable body weight changes Saline treatment permitted good growth The food intake is nevertheless lower than for *ad libitum* fed animals Pair fed animals showed a slightly higher body weight gain than adrenalectomized animals receiving saline therapy

B *Experiments designed to Test the Stability of Liver and Muscle Lysosomes*

Enzyme release in homogenates The data presented in Table II indicate that adrenalectomy results in enhanced release of all the four enzymes studied Saline-

TABLE II Unsedimentable acid hydrolase activity in liver and muscle homogenates of untreated saline treated corticosterone treated adrenalectomized animals and intact control animals

Animal group	Cathepsin D (%)	β glucuronidase (%)	Aryl sulphatase (%)	Acid RNase (%)
Liver				
Adx — untr	(6) 9.0 ± 0.5	(6) 12.2 ± 1.1	(4) 16.5 ± 0.8	(4) 17.1 ± 2.7
Pair fed	(6) 3.3 ± 1.6[c]	(6) 7.8 ± 0.8[c]	(4) 12.2 ± 1.1[b]	(4) 11.7 ± 1.4[c]
Ad lib fed	(6) 4.1 ± 0.6[c]	(6) 7.4 ± 1.0[c]	(4) 8.4 ± 0.6[c]	(4) 11.1 ± 1.1[c]
Adx + 300 µg B	(5) 3.8 ± 1.3[c]	(5) 7.0 ± 0.6[c]		
Adx + 3 mg B	(3) 0.3 ± 0.3[cb]	(3) 2.6 ± 1.9[ca]		
Adx — saline	(5) 9.8 ± 0.8	(7) 12.3 ± 2.2	(4) 14.0 ± 1.4	(4) 10.0 ± 1.4
Pair fed	(5) 4.1 ± 1.3[c]	(7) 7.2 ± 1.0[b]	(4) 8.4 ± 0.6[b]	(4) 8.2 ± 2.4
Muscle				
Adx — untr	(6) 28.3 ± 1.9	(6) 34.6 ± 2.3	(4) 36.2 ± 0.6	(4) 32.5 ± 1.0
Pair fed	(6) 22.1 ± 1.5[b]	(6) 27.7 ± 1.5[a]	(4) 27.5 ± 1.8[c]	(4) 19.9 ± 2.8[c]
Ad lib fed	(6) 19.0 ± 1.6[c]	(6) 23.3 ± 1.2[b]	(4) 30.9 ± 0.6[c]	(4) 25.8 ± 0.7[c]
Adx + 300 µg B	(5) 18.9 ± 1.3[c]	(5) 24.0 ± 1.5[b]		
Adx + 3 mg B	(5) 18.0 ± 1.2[c]	(5) 16.5 ± 2.6[c]		
Adx — saline	(5) 22.6 ± 4.1	(5) 31.8 ± 2.6	(4) 30.1 ± 2.5	(4) 27.0 ± 2.7
Pair fed	(5) 15.7 ± 3.6[b]	(5) 19.5 ± 3.2[b]	(4) 27.5 ± 1.8	(4) 21.7 ± 2.9[b]

Values represent means ± standard errors of means Unsedimentable activities are calculated as per cent of total activities in whole homogenates (cathepsin β glucuronidase or RNase) or as per cent of the sum of activities in soluble and particulate fractions (Aryl sulphatase) Number in parentheses indicate number of animals a b or c = lower than adx a $p < 0.10$ b = $p < 0.05$ c $p < 0.01$ cb ca = lower than adx and adx + 300 µgm B Adx = adrenalectomized Untr untreated Saline saline treated Adx + 300 µg B adx receiving 300 µg corticosterone daily for 5 days Adx + 3 mg B adx receiving 3 mg corticosterone daily for 5 days Pair fed = pairfed to adx

treated adrenalectomized animals show values for unsedimentable activities that are comparable to those of untreated adrenalectomized rats No significant differences were observed between *ad libitum* fed animals and animals pair fed to untreated experimentals It is furthermore evident that the relative unsedimentable activity of hydrolases in muscle homogenates is 2—3 times higher than in liver homogenates when tissues from the same group of animals are compared Administration of 300 µg of corticosterone daily to adrenalectomized animals decreased the unsedimentable activities of β glucuronidase and cathepsin D to values not significantly different from those of intact controls Table II also demonstrates the effect of administering 3 mg corticosterone daily to adrenalectomized animals The data suggest that the later treatment leads to a further reduction in unsedimentable enzyme in liver and muscle as compared to animals receiving only 300 µg of the hormone daily

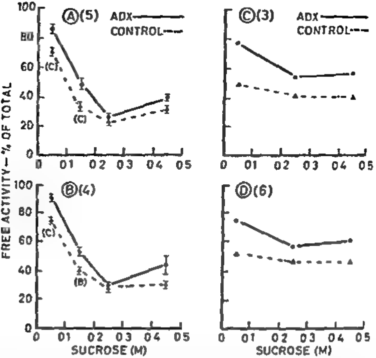

Fig 1 Osmotic fragility of liver and muscle lysosomes Lysosomerich liver and muscle fractions were preincubated at different sucrose concentrations at 0° C for 30 min After readjusting the sucrose concentrations to the original value 0 25 M free β glucuronidase activity was measured in the different fractions Incubation times for the assay were 10 min for liver and 20 min for muscle fractions The incubation medium contained 0 25 M sucrose for osmotic protection of the particles

A Osmotic fragility of hepatic lysosomes from untreated adrenalectomized (adx) and pair fed control animals

B Osmotic fragility of hepatic lysosomes from saline treated adrenalectomized rats and pair fed controls

C and D Osmotic fragility of muscle lysosomes prepared from untreated (C) and saline treated (D) adrenalectomized rats and pair fed controls Units on the ordinate are the same as in A and B Free β glucuronidase activity is expressed as per cent of total activity i e activity in fractions containing 0 2 per cent (v/v) Triton X 100 Number in parentheses indicate number of experiments Letters in parentheses are defined as in Table II

In vitro release of hydrolases from liver and muscle lysosomes

The result of measuring released β glucuronidase in liver and muscle fractions that had been exposed to different sucrose concentrations are demonstrated in Fig 1 The data show that a lowering of the osmolarity releases more of the enzyme from liver and muscle lysosomes of untreated and saline treated adrenalectomized animals than from particles prepared from intact control animals The increased osmotic fragility of muscle lysosomes from adrenalectomized animals is also indicated in Table III where released activity in fractions kept in an isosmotic medium (0 25 M) is subtracted from that of fractions exposed to hypo- or hyperosmotic media The results also show that hepatic lysosomes are more sensitive to a change in osmolarity than particles from muscle

TABLE III Effects of osmotic stress on free β-glucuronidase activity in mitochondrial muscle fractions from untreated and saline treated adrenalectomized animals and from pair fed control animals

		Per cent free β glucuronodase activity Adx	Control
Untreated adx			
Activity in 0.25 M sucrose	(3)	54.0±3.0	40.6±2.5[b]
Increase in 0.05 M sucrose	(3)	21.1±2.9	9.0±3.0[b]
Increase in 0.45 M sucrose	(3)	3.0±1.1	0.0±1.6
Saline Treated adx			
Activity in 0.25 M sucrose	(6)	57.8±4.6	45.2±5.2
Increase in 0.05 M sucrose	(6)	15.8±1.4	6.2±0.9[c]
Increase in 0.45 M sucrose	(6)	3.2±2.2	0.50±3.1

Values represent means of measurements ± standard errors of the means. b, c: higher in experimental fractions than in controls; b: $p < 0.05$, c: $p < 0.01$. Number in parentheses indicate number of experiments.

TABLE IV

A Concentration dependent release of β glucuronidase by vitamin A alcohol in muscle and liver fractions

		Concentration of vitamin A 10^{-3} M	5×10^{-4} M	10^{-4} M	O M
Liver fractions	(4)	79.2±3.2	41.0±3.0	8.3±3.3	5.2±0.9
Muscle fractions	(3)	61.3±2.4	40.7±3.6	26.0±3.0	21.7±2.9

B Inhibition of β-glucuronidase activity by vitamin A alcohol in muscle and liver fractions

		Incubation time minutes	10^{-3} M	Concentration of Vitamin A 5×10^{-4} M	O M
Muscle	(4)	0	67.2±3.1	81.5±1.5	100.0
	(4)	60	73.7±7.9	82.8±2.1	100.0
Liver	(3)	0	77.0±3.0		100.0
	(3)	30	80.0±4.0		100.0
	(3)	60	79.2±7.6		100.0

Values represent means ± standard errors of means. Number in parentheses indicate number of measurements.

A. Values represent increases in unsedimentable β glucuronidase after 60 min of incubation with vitamin A and are calculated as per cent of total activity in fraction, i.e. sum of activity in *soluble and particulate fractions*.

B. Total β glucuronidase activity is presented as per cent of activity in aliquots not treated with vitamin A.

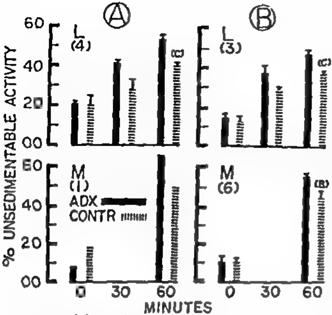

Fig. 2. *In vitro* release of unsedimentable β-glucuronidase activity in presence of 5×10^{-4} M vitamin A alcohol. Lysosome-rich liver and muscle fractions were incubated in presence of vitamin A for the time intervals indicated below the columns. The fractions were then recentrifuged and β-glucuronidase activity determined in soluble and particulate fractions. Particulate fractions were pretreated with Triton X-100 (0.2 per cent v/v) before the enzyme assay. Unsedimentable activity is given as per cent of the sum of activities in unsedimentable and sedimentable fractions.

A. *In vitro* release of β-glucuronidase from liver (L) and muscle (M) fractions from untreated adrenalectomized and pair-fed control animals.

B. *In vitro* release from fractions prepared from saline-treated experimentals and pair-fed controls. Number in parentheses indicate number of experiments. Letters in parentheses are defined as in Table II. Standard errors are indicated in the graph.

In preliminary experiments the ability of vitamin A alcohol to release β-glucuronidase from normal liver and muscle lysosomes was tested. It was found that the effect of the vitamin was comparable in the two tissues, and that the increases in unsedimentable (released) enzyme after 60 min of incubation with 5×10^{-4} M vitamin A were about 40 percent of total activity for both tissues (Table IV). It was decided to use this concentration of the vitamin in subsequent experiments where the labilizing effect of vitamin A alcohol was tested on particles from adrenalectomized and control animals. The results of these experiments (Fig. 2) indicate a greater release of β-glucuronidase from tissue fractions isolated from untreated and saline-treated experimentals. Thus, after 1 hr of incubation with the vitamin the unsedimentable activity is about 50 percent of total activity in both liver and muscle fractions from adrenalectomized animals as compared to 40 percent in control fractions from both tissues. There appeared to be no significant difference between experimental and control fractions at time zero. In studying the effect of different concentrations of vitamin A on the stability of liver and muscle lysosomes it was also found that the vitamin induces a significant decrease in the total activity of β-glucuronidase. This inhibition was particularly evident in muscle fractions where the total activity in fractions incubated in presence of 10^{-3} M vitamin A was about 70 percent of the activity in control fractions. The inhibitory effect was dependent on the concentration of the vitamin, but independent of incubation time, as indicated in Table IV.

In vitro release of enzymes from liver and muscle lysosomes exposed to pH 5 was

TABLE V *In vitro* release of unsedimentable and free β glucuronidase from hepatic fractions incubated at pH 5

			Incubation time in minutes	
			0	60
Untreated adx				
unsedimentable	adx	(9)	4 7±1 2	37 8±2 3
	control	(9)	7 2±2 6	58 8±4 4[c]
free	adx	(5)	19 6±1 9	50 6±4 6
	control	(5)	19 0±2 3	62 0±7 0[b]
Saline treated adx				
unsedimentable	adx	(4)	5 5±0 3	48 5±2 2
	control	(4)	5 4±0 9	61 3±1 9[c]
free	adx	(3)	21 8±2 3	44 9±3 0
	control	(3)	19 0±3 0	61 1±2 9[b]

Values represent means ± standard error means b $p < 0\ 05$ c $p < 0\ 01$

Unsedimentable or free activities are calculated as per cent of total activity in the fractions

Number in parentheses indicate number of animals

first followed by measuring β glucuronidase activity rendered unsedimentable or free at different incubation times There was no significant differences in the rate of release from muscle lysosomes prepared from intact controls or from untreated or saline treated adrenalectomized animals The results obtained on liver fractions showed in contrast to data from experiments with osmotic stress and incubation with vitamin A that less β glucuronidase was released from hepatic fractions of adrenalectomized animals as compared to intact controls This holds true whether the release is measured as free or unsedimentable activity The difference in release shows up particularly well after 60 min of incubation and in Table V are summarized the results obtained for enzyme release after 0 and 60 min of incubation at pH 5 It can be seen that more enzyme is released after one hour of incubation from control fractions as compared to fractions prepared from untreated or saline treated adrenalectomized animals There appeared to be no significant difference in *in vitro* release initially between experimental and control fractions Additional experiments on *in vitro* release from hepatic fractions exposed to low pH were done by measuring unsedimentable aryl sulphatase and acid RNase at different incubation times The data obtained were essentially the same as those for β glucuronidase in that less enzyme was released from fractions from experimental animals

C *Subcellular localization of acid hydrolases*

To obtain information about sedimentation properties of hepatic lysosomes liver homogenates from adrenalectomized and intact animals were fractionated into 5 fractions by differential centrifugation (de Duve *et al* 1955) The distribution of 4 enzymes in hepatic fractions is demonstrated in Fig 3 The histogram is prepared

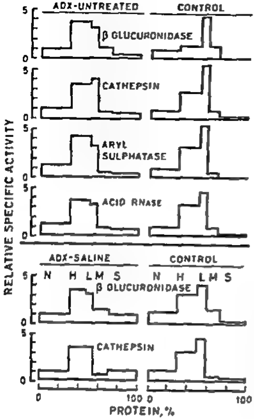

Fig 3 Frequency distribution of some acid hydrolases in 5 subcellular liver fractions from untreated and saline treated adrenalectomized rats and pair-fed control animals Abscissa fractions are represented by their relative protein content, in the order in which they were isolated, *i e*, from left to right N, nuclear, M, heavy mitochondrial, L, light mitochondrial, M microsomal, and S final supernatant fraction Ordinate mean relative specific activity of fractions

according to de Duve *et al* (1955) When enzyme distribution in homogenates from adrenalectomized and intact animals are compared, two differences may be pointed out First there is a relatively higher proportion of soluble enzyme in homogenates prepared from adrenalectomized animals as compared to controls This holds true for both untreated and saline treated animals Secondly enzymes associated with particulate fractions are more concentrated in the heavier fractions in homogenates from adrenalectomized animals as compared to controls Thus, for normal liver, highest relative specific activity is found in the light mitochondrial fraction In liver homogenates from adrenalectomized rats, the highest specific activity occurs in the heavy mitochondrial fraction Therefore, if one assumes that particle density is unchanged in experimental and control homogenates the average size of the particles must have increased as a result of adrenalectomy The changes observed are also roughly parallel for the enzymes studied

D *Enzyme activities in muscle and liver*

Measurements of the relative proportions of soluble and sedimentable enzyme in liver and muscle homogenates necessitated determinations of total enzyme activity, and since significant differences were observed in enzyme activities as a result of

TABLE VI Hydrolase activity in liver and muscle of untreated saline treated and corticosteron treated adrenalectomized rats

Animal group		Cathepsin D		β glucuronidase		Aryl sulphatase		Acid ribonuclease
Liver								
Adx untreated	(7)	12.7±0.4	(7)	40.4±1.5	(4)	59.6±6.1	(6)	32.7±2.9
Pair fed	(7)	10.2±0.6[b]	(7)	27.2±1.2[c]	(4)	50.1±0.6	(6)	27.2±0.5[c]
Ad lib fed	(7)	9.2±0.5[c]	(7)	26.1±1.7[c]			(6)	14.8±0.9[cc]
Adx+300 μgB	(7)	10.0±0.5[b]	(7)	30.5±2.3[b]				
Adx+3 mgB	(5)	7.7±0.1[cc]	(3)	23.3±1.5[bb]				
Adx saline	(4)	11.3±0.7	(11)	40.5±1.5	(6)	62.9±2.6	(7)	29.2±1.3
Pair fed	(4)	9.7±0.6	(11)	31.1±1.8[c]	(6)	55.9±3.4[b]	(7)	24.4±1.4[b]
Muscle								
Adx untreated	(7)	11.6±0.4	(6)	6.4±0.4	(6)	10.8±0.9	(6)	20.0±0.9
Pair fed	(7)	8.2±0.4[c]	(6)	4.1±0.2[c]	(6)	8.6±0.04	(6)	17.1±0.4[b]
Ad lib fed	(7)	8.4±0.4[c]	(6)	4.5±0.3[c]	(6)	8.1±0.1	(6)	14.8±0.9[c]
Adx+300 μgB	(6)	8.2±0.6[c]	(6)	4.0±0.4[b]				
Adx + 3 mg B	(5)	6.6±0.4[cb]	(5)	3.3±0.4[c]				
Adx saline	(4)	11.4±0.2	(8)	5.2±0.4	(6)	11.3±0.1	(6)	20.7±2.8
Pair fed	(4)	8.2±0.2[c]	(8)	3.5±0.3[c]	(6)	7.5±0.2[c]	(6)	16.9±3.0[c]

Values represent means ± standard errors of means. Numbers in parentheses indicate number of animals in each group. Specific activities: Cathepsin D, μg tyrosine/mg protein/60 min (muscle) or 10 min (liver). β glucuronidase, μg phenolphthalein/mg protein/60 min (muscle) or 10 min (liver). Aryl sulphatase, 10^{-4} × μM nitrocatechol/mg protein/60 min (muscle) or 10 min (liver). RNase, 10^{-3} × O.D./mg protein/60 min (muscle) or 10 min (liver). a, b, c, cc and bb defined as in Table II.

adrenalectomy, some of these data will be presented. Table VI shows enzyme activities of some hydrolases in liver and muscle of untreated and saline treated adrenalectomized rats and in intact control animals. The data indicate that adrenalectomy results in a roughly parallel enhancement in activity of ribonuclease, cathepsin D, β glucuronidase and aryl sulphatase in liver and muscle. Both pair-fed and *ad libitum* fed animals served as controls for untreated adrenalectomized animals and it can be noted that pair fed animals show values of liver enzymes that are intermediate between those of adrenalectomized and *ad libitum* fed controls. This suggests a starvation effect since the food intake is lower in pair fed than in *ad libitum* fed animals. It is furthermore evident that administration of 300 μg corticosterone to adrenalectomized animals leads to a reduction in activity of cathepsin D and β glucuronidase in muscle and liver to values not significantly different from those of pair fed intact animals. Administration of a higher dose of hormone results in further reductions in the activities of these two enzymes in both tissues.

To assess the amount of enzyme in liver and gastrocnemius muscle, organ weights were recorded, and total amount of enzyme was calculated as enzyme activity per

TABLE VII Total cathepsin activity in muscle and liver of untreated, saline treated and corticosterone treated adrenalectomized rats

Animal group		Cathepsin D (%)
Liver		
Adx untreated	(4)	104 1 ± 9 0
Pair fed control	(4)	100 0
Adx + 300 μg B	(4)	98 1 ± 8 8
Adx + 3 mg B	(4)	101 9 ± 6 6
Adx saline	(7)	99 5 ± 13 0
Pair fed control	(7)	100 0
Muscle		
Adx untreated	(4)	155 0 ± 16 0[b]
Pair fed control	(4)	100 0
Adx + 300 μg B	(4)	92 6 ± 6 0
Adx + 3 mg B	(4)	60 4 ± 2 2[c]
Adx saline	(7)	124 6 ± 7 3[b]
Pair fed control	(7)	100 0

Values represent means ± standard errors of means Total activities in organs are calculated in terms of per cent of total activities in organs of intact pair fed controls Number in parentheses indicate number of animals a B and c = different from pair fed controls a = p < 0 10 b = p < 0 05, c = p < 0 01

gm of tissue times the organ weight Changes in total activities were essentially similar for the different enzymes and the results obtained for cathepsin D may serve as an example In Table VII total cathepsin activity in pair fed controls was set to unity and activities in organs of experimental animals were calculated as per cent of the control values The results show slight differences in total hepatic enzyme activity between the different groups of animals The observed differences in activity as a result of adrenalectomy therefore reflect a concentrating of enzyme in the organ in cases where tissue wasting takes place This indicates a relative sparing of lysosomal enzyme proteins since the concentration of proteins was found to remain unchanged The situation in muscle was different in that a distinct increase in total activity of all four hydrolases was evident in both untreated and saline treated adrenalectomized animals Administration of corticosterone resulted in a dose dependent decrease in total muscle hydrolase activity Administration of 300 μg of corticosterone daily to adrenalectomized animals decreased total enzyme activity to values comparable to those of intact pair fed animals Treatment with 3 mg of the hormone daily reduced the activities significantly below control values Concurrently with this decreased enzyme activity a considerable loss of muscle mass occurred Thus the average gastrocnemius weight in rats given 3 mg corticosterone daily was 0 26 g as compared to 0 38 g in pair fed control rats

Discussion

Earlier studies (Weissmann 1965, Bird *et al* 1968) have shown that high doses of certain glucocorticoids will stabilize lysosomes The present investigation demonstrated the effects of a reduction of the endogenous glucocorticoid levels on lysosomal stability Several of the physiological changes observed in the adrenalectomized rat may be secondary effects of adrenal insuffiency Thus the present study shows as do earlier investigations, that adrenalectomy leads to reduced food intake, resulting in subnormal growth The untreated adrenalectomized animals also develop electrolyte imbalance These two factors have been taken into consideration in the current studies by using pair fed control animals in all experiments and by studying the effects of saline maintenance therapy

The following parameters relevant to lysosomal stability have been studied (1) unsedimentable enzyme in freshly prepared homogenates and (2) the effects of osmotic stress, vitamin A and low pH on *in vitro* release of lysosomal hydrolases The decreased latency of enzymes in homogenates and tissue fractions from adrenal ectomized animals may be the result of an *in vivo* released of enzymes and/or more mechanically fragile particles A better indication of *in vivo* release may be to measure serum levels of enzymes Janoff *et al* (1962) and Kovanic (1968) studied changes in serum levels of lysosomal hydrolases in normal and adrenalectomized rats exposed to different shocks Their data indicate that serum levels of enzymes in unstressed adrenalectomized rats are comparable to those of intact animals The decreased latencies of hydrolases found in the present study in homogenates from adrenalectomized animals therefore indicate that lysosomes from adrenalectomized animals are more liable to rupture during the homogenization procedure The much higher unsedimentable activities found in muscle than in liver homogenates probably reflect the rougher homogenization required for grinding of muscle The changes in mechanical fragility and/or *in vivo* release brought about by adrenalectomy are not corrected by saline treatment but administration of corticosterone is effective in reducing unsedimentable enzyme activity These results indicate that the increased fragility of particles results from a lack of glucocorticoids In support of this, Beaufay *et al* (1959) also observed slight decreases in unsedimentable lysosomal enzymes in liver of rats treated with 12,5 mg hydrocortisone daily for 3 days Starvation has been found to render enzymes unsedimentable in rat liver homogenates (Beaufay *et al* 1959, Dingle *et al* 1966 Berg 1968) The observed increases of released enzyme in homogenates from adrenalectomized animals is not likely to be a starvation effect since pair fed control animals showed significantly lower values for unsedimentable enzyme Other studies done in this laboratory also indicate that increased release is evident only in tissues from animals that are in a more severe state of starvation than our adrenalectomized animals (to be published)

The increased sensitivity to osmotic stress and vitamin A alcohol observed with particles of adrenalectomized animals also lends support to the concept of lysosomal stabilization by adrenal steroids Earlier studies (Weissmann 1965) have clearly

shown an antagonizing effect of lysosomal stability between glucocorticoids and vitamin A both *in vivo* and *in vitro* However, there is a discrepancy between the results obtained with osmotic stress and vitamin A on one hand and the effects of exposing hepatic lysosomes to low pH on the other *In vitro* release of enzyme at pH 5 was distinctly slower in liver lysosomes prepared from adrenalectomized animals In contrast to this de Duve *et al* (1962) observed *in vitro* stabilization by glucocorticoids of rat liver lysosomes exposed to pH 5 The sensitivity of hepatic lysosomes of adrenalectomized animals to osmotic stress and low pH are reminiscent of results reported by Wattiaux *et al* (1963) on liver lysosomes prepared from rats injected with *Triton WR 1339* They too found that these particles were more sensitive to osmotic stress and less sensitive to low pH than normal particles They suggested that both responses may be related to the loading of particles with detergent The resultant increase in particle size would render the lysosomes more sensitive to osmotic stress, and the uptake of non enzymatic material might somehow interfere with the thermal motions of lysosomal cathepsins that are postulated to be involved in hydrolysis of the membrane of their host particles The increased osmotic fragility of particles from liver of glucagon treated rats has also been explained to result from concommitant increase in particle size (Deter and de Duve 1967)

As discussed above the increased release of enzymes observed in tissue homogenates of adrenalectomized animals may result from an increased mechanical fragility rather than an *in vivo* release of hydrolases An increased mechanical fragility may also be correlated with increased particle size since a larger particle will be more liable to damage by the shearing forces generated by the velocity gradient in the homogenizer and by compression between the walls of the tube and the pestle (Deter and de Duve 1967) *We would like to propose that the changes in lysosomal* stability observed in muscle and liver as a result of adrenalectomy in the present investigation are indicative of an increased particle size This contention is also supported by the finding of a higher proportion of particles sedimenting at low speed in hepatic homogenates from adrenalectomized animals as compared to intact pair fed controls Glucocorticoids have been reported to inhibit pinocytosis and phagocytosis (Dougherty 1961) Since secondary lysosomes arise by a fusion between pre existing lysosomes and pino- or phagocytic vacuoles an inhibition of these processes *might result in a decreased particle size An increase in particle size* would result if removal of glucocorticoids stimulates phago or pinocytosis

The increases observed in total enzyme activity in liver as a result of adrenalectomy are similar to those reported in starving animals (Beaufay *et al* 1959 Berg 1968 Bird *et al* 1968) and are typical for tissue degeneration in general in that a relative sparing of lysosomal enzyme proteins occurs (de Duve 1963)

While no significant changes were found for total enzyme activity in liver of adrenalectomized animals marked increases were found in total muscle acid hydrolase activity Administration of high doses of corticosterone resulted in decreases of both specific and total enzyme activities There thus seemed to be an inverse relation between corticosterone levels and muscle hydrolase activity *This finding* was un

expected since glucocorticoids in high doses induce necrotic changes in skeletal muscle (Smith 1964), and in all studies reported so far, necrosis has consistently been associated with increased activity of acid hydrolases

The observed increases in muscle hydrolases in adrenalectomized animals are probably not due to decreased food intake Recent experiments done in this laboratory have shown that starvation may lead to decreased specific and total activity of muscle ribonuclease Buchanan and Schwartz (1967) did not find significant differences in β glucuronidase and acid phosphatase activity in skeletal muscle of rats injected with cortisone This discrepancy may be due to the fact that they measured their activities in fractions remaining soluble after sedimentation of a nuclear fraction They also reported that activities were so low that no attempt was made to assay cathepsin D We have found that the nuclear fraction in muscle homogenates prepared in 0 25 M sucrose contains approximately 70 percent of the total acid hydrolase activity The difference in procedure makes it difficult to make any comparison

Histochemical methods have failed to detect lysosomes in normal muscle fibers of rats Price *et al* (1964) have described what probably are lysosomes in macrophages but not in normal fibers of rat skeletal muscle Similar results are reported by Smith (1964) This author also did histochemical studies of muscle in rats treated with the glucocorticoid triamcinolone She found that macrophages were absent in muscle tissue from animals treated with the hormone Preliminary investigations in this laboratory also indicate that macrophages are absent in muscle of rats treated with 5 mg of corticosterone daily for 5 days We therefore believe that the observed changes in acid hydrolase activity reflects an effect of adrenal steroids on the connective tissue component muscle

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References

ANSON M L The estimation of cathepsin with hemoglobin and the partial purification of cathepsin *J gen Physiol* 1937 *20* 565—574

BEAUFAY H E VAN CAMPENHAUT and C DE DUVE Tissue fractionation studies 11 Influence of various hepatotoxic treatments on the state of some bound enzymes in rat liver *Biochem J* 1959 *73* 617—623

BERG T *Studies of lysosomal hydrolase*
Ph D Thesis Rutgers — The State

BIRD J W C T BERG and J H LEA
of adrenalectomized rats *Proc Soc e*

BUCHANAN W E and T B SCHWARTZ Lysosomal enzyme activity in heart and skeletal muscle of cortisone treated rats *Amer J Physiol* 1967 *212* 732—736

DE DUVE C General properties of lysosomes The lysosome concept In *Ciba Foundation Symposium on Lysosomes* Ed by A V S de Reuck and M P Cameron Little Brown Boston 1963 1—31

DE DUVE C and H BEAUFAY Tissue fractionation studies 10 Influence of ischaemia on the state of some bound enzymes in rat liver *Biochem J* 1959 *73* 610—616

DE DUVE C B C PRESSMAN R GIANETTO R WATTIAUX and F APPELMANS Tissue

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From the Department of Pharmacology, University of Uppsala, Sweden

Autoradiographic Studies with ³⁵S-Sulfate on Somatotrophin and Estrogen Sensitivite Growth Zones in Rat and Mouse Costal Cartilage

By

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Abstract

HERBAI, G. *Autoradiographic studies with ^{35}S sulfate on somatotrophin and estrogen sensitive growth zones in rat and mouse costal cartilage.* Acta physiol. scand. 1970. 79. 351—358.

An autoradiographic study was performed on the regional pattern of incorporation of ^{35}S-sulfate in different anatomical regions of costal cartilage in growing mice and rats *in vivo* and *in vitro*. Profound regional differences in sulfate incorporation rate have been revealed between the medial or "resting" part of costal cartilage and the "active" growing region of the rib which is located in close association with the osteochondral junction. The regional pattern [illegible]

for 10 days restored the rate of sulfate uptake to above normal levels in the growth zones of the ribs. A 10 day treatment with a daily dose of 100 µg estradiol benzoate per mouse caused a pronounced fall in the sulfate incorporating activity of the growth region and markedly narrowed the "active" zone of the costal cartilage.

Cartilage tissue from different animals species is frequently used in many fields of biochemical and metabolic research. Rat and calf costal cartilage has been used for studies on the biosynthesis of chondroitin sulfate and protein-mucopolysaccharide complex (Gross, Mathews and Dorfman 1960, Kleine and Hilz 1968). A large amount of work has been done on the growth hormone dependent "sulfation factor" and most of the results have been achieved by using ^{35}S-sulfate incorporation techniques into rat costal cartilage (Salmon and Daughaday 1957, Denko and Bergenstal 1961, Almqvist 1961, Daughaday and Kipnis 1966). Whitehouse and Bostrom (1961 and 1965) also used rib cartilage form calf and rat in their studies on the effects of antiinflammatory drugs upon the biosynthesis of mucopolysaccharides. In several investigations costal cartilage from rabbits (Katura and Davidson 1966), guinea pigs (Antonowicz and Kodicek 1962), mice (Pelc and Glucksmann 1955)

also from puppies (Bowness and Jacobs 1968) has been used for different purposes

In all of the studies mentioned above the costal cartilage was assumed to be a metabolically homogeneous tissue without regional differences in chondroitin sulfate synthetizing activity No attention was paid to the possible occurrence of growth zones in the cartilage samples Very little is written in the literature about the anatomical location from which the cartilage tissue was taken for use in the experiments

The distribution pattern of ^{35}S sulfate has been previously demonstrated using autoradiographic techniques in cartilage tissue taken from different organs (Belanger 1954, Bostrom and Jorpes 1957, Campo and Dziewiatkowski 1963, Engfeldt and Westerborn 1960) There is, however, very little published information about the regional pattern of sulfate uptake in different regions of mouse and rat costal cartilage

Howell *et al* (1960) have shown marked differences in cell volume and electrolyte profile between cartilage slices derived from the osteochondral junction and from medial parts of calf rib Recently Yde (1968) has described a modified technique for determination of the sulfation factor in intact rats Since the costal ends of the cartilage pieces incorporated more $^{35}SO_4$ than the remaining parts, they were consistently eliminated and discarded from the study

The present work is an introduction to a larger project concerned with quantitative studies of sulfate incorporation into costal cartilage under the influence of different drugs and hormones In order to clarify which parts of the cartilage tissue are most suitable for further studies, the regional incorporation pattern of ^{35}S sulfate in different parts of rat and mouse rib cage was investigated with Ullbergs autoradiographic technique (Ullberg 1954) After demonstration of intensely active growth zones at the osteochondral junction both *in vivo* and *in vitro*, the sensitivity of this region to growth hormone and estrogens were also investigated, since both these hormones exert marked and opposite metabolic effects on cartilage metabolism and growth

Material and Methods

Growing female Sprague Dawley rats weighing about 60 g and growing female N M R I mice weighing about 16 g were obtained from Anticimex AB Norrviken Sweden The animals were housed in plastic cages and they were fed ad libitum with Anticimex pellets (no 210 for rats and no 213 for mice They were allowed free access to tap water The temperature of the room was kept at 25° C and a normal 12 12 day night rhythm was maintained For the mouse-embryo experiments mice were used at a late stage of pregnancy)

Hypophysectomy of the mice was performed by a modification (Herbai, to be published) of the transauricular approach Falconi and Rossi 1964) The animals were used for the experiments 20 days after the operation and their sella region was carefully examined at autopsy for pituitary remnants using 16 × magnification Estradiol benzoate (Organon Co Oss Holland) was dissolved in olive oil and injected s.c. in a daily dose of 100 µg per animal for 10 days prior to the experiment Ovine growth hormone NIH GH S8[1] was dissolved in 5 % glucose adjusted to pH 9 5 by sodium hydroxide and injected i p in a dose of 500 µg per animal daily for 10 days prior to the experiment

In the in vivo experiments ^{35}S labeled sodium sulfate (the Radiochemical Centre Amers

[1] (The preparation was kindly supplied by the National Institute of Health, Endocrinology Study Section Bethesda Maryland U S A

Fig 1

Fig 2

Fig 1 Distribution pattern of ^{35}S sulfate in a mouse fetus 8 hrs after i v injection of the isotope to a pregnant mouse Paravertebral sagittal section Intense isotope accumulation is visible at the cross sections of the vertebral transverse processes the dorsal and the ventral costal segments

Fig 2 Autoradiogram of a frontally sectioned flattened rib cage from a growing immature rat Inhomogeneous ^{35}S incorporation at 4 hrs is seen in the sternum costal cartilage and the osteochondral junction

ham England) was injected into each animal i v by the orbital plexus technique (Pinkerton and Webber 1964) in a dose of 10 μC/10 g b w with 10 μg of carrier sodium sulfate Four hours later the animals were killed by ether anesthesia and their rib cages were dissected free from adherent tissues in situ The rib cage was then removed by cutting the bony ribs at their vertebral attachment and the whole sample was then placed into 50 ml isotonic sodium sulfate solution for 24 hrs In the *in vitro* experiments the dissected rib cages together with the inter costal muscles were incubated in stoppered Erlenmeyer flasks with Krebs Ringer phosphate

isotonic Na_2SO_4 solution for 24 hrs From this stage the same procedure was followed in the *in vivo* as in the *in vitro* experiments The rib cages were flattened between two metal plates

Results

Pregnant mice were injected with ^{35}S sulfate and killed ■ hrs later The distribut of ^{3} S sulfate in the fetal body is shown in Fig 1 Dorsal and ventral cross s

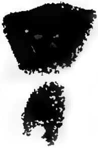


Fig 3

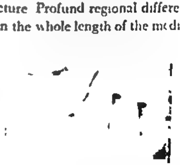
Fig 5

Fig 3 Seventy-fold magnification of the osteochondral region of a rib taken from a growing rat which has been given ^{35}S sulfate Incorporation time 4 hrs

Fig 5 Autoradiogram of costal cartilage including the osteochondral region after *in vitro* incubation of a mouse rib cage with ^{35}S sulfate for 2 hrs and washing for 24 hrs

of the rib cage can be seen on the picture with rather intense sulfate incorporation into both parts of the developing ribs

The incorporation pattern of in vivo injected ^{35}S sulfate in different parts of rat rib cage is demonstrated in Fig 2, which shows a frontal section of the sample through the sternum and along the flattened ribs Transversal lines of increased sulfation activity are seen in the sternum The sulfate uptake into the bony parts of the ribs is so low that it is invisible in the picture Profund regional differences are visible in the intensity of sulfate uptake between the whole length of the medial "rest-



'ate sulfate in frontally sectioned flattened
ws narrow transversal growth zones and
the growth zone at the osteochondral
costal cartilage Incorporation time 4 hrs

Fig 6 Autoradiographic visualization of the sulfate incorporation pattern in the osteochondral region of the mouse rib 4 hrs after $^{35}SO_4$ injection to a) normal mouse b) hypophysectomized mouse c) hypophysectomized mouse after growth hormone treatment d) estradiol benzoate treated mouse

The figures were obtained using exactly equal autoradiographic and photogr ditions

ing' part of cartilage and the lateral 'active' region close to the osteochondral junction. The strong accumulation indicates the presence of growth zones with intense chondroitin sulfate synthesis. This region of the rib is probably the site of longitudinal growth corresponding to the epiphysis region of long bones. Further magnification of the osteochondral junction of a rat rib is made in Fig. 3 and it can be seen that the growth area consists of complex tissue pools with varying sulfation activity. The incorporation of *in vivo* injected ^{35}S sulfate into different parts of mouse costal cartilage which is demonstrated in Fig. 4, is very similar to that seen in the rat. It is seen from both Fig. 2 and 4 that the sulfate uptake in the remaining 'resting' part of costal cartilage is rather constant along the cartilage with accentuated uptake in the surface areas.

When a rib cage dissected from a normal growing mouse is incubated with ^{35}S sulfate *in vitro*, a strong accumulation of the isotope occurs around the osteochondral region as is seen in Fig. 5. It is obvious that the high sulfation activity of the costal growth zone is also maintained *in vitro*.

The effects of hypophysectomy with and without growth hormone administration on the area around the osteochondral junction were also investigated. Fig. 6 a and b demonstrates that the activity of the osteochondral junction characteristic for normal growing mice is almost completely abolished in hypophysectomized animals. The operated mice showed a postoperative growth arrest with instantaneous plateauing of the body weight. Growth hormone treatment in hypophysectomized animals during 10 days before the isotope experiment counteracted the effect of hypophysectomy (Fig. 6 c). Rather strong sulfate incorporation became visible at the growth zones of the somatotrophin treated animals and the intensity of sulfate uptake seemed in fact to be stronger than that in the cartilage of normal animals. Finally the effect of estrogen treatment on the cartilage growth zone of normal mice was investigated since it is generally known that estrogenic substances cause growth retardation in several animal species (Silberberg and Silberberg 1956). Fig. 6 d shows the rib growth zone of an estradiol benzoate treated animal: it is narrow with a marked decrease in sulfate incorporating capacity.

Discussion

In spite of the frequent utilization of costal cartilage from different animal species in experimental metabolic research, the literature contains only sporadic data about differences in the sulfation pattern between different parts of rib cartilage (Klebanoff *et al.* 1958, Yde 1968). In most of the studies which have been performed by techniques using isotope incorporation into costal cartilage the anatomical regional origin of the cartilage samples is not exactly defined. It has been shown by many investigators in several animal species that *in vivo* injected ^{35}S sulfate is retained in cartilage tissue mainly in organically bound form in the mucopolysaccharides and that the intensity of sulfate incorporation reflects the biosynthesis rate of chondroitin sulfate (*vide* introduction). It can therefore be assumed that the amount of bound

radioactivity in the cartilage samples of this study gives a good indication of the rate of condroitin sulfate synthesis

The mouse fetus autoradiogram shows marked sulfate accumulation in all rib cross-sections indicating mucopolysaccharide synthesis involving even the dorsal part which becomes bone with diminished sulfation activity post partum The fetal sulfate distribution is similar to that observed in rabbits by Boström and Jorpes (1957)

The autoradiograms from growing animals show that the bony parts of the ribs have a very low sulfate incorporating activity which probably depends on the slow mucopolysaccharide turnover of the mature bone tissue On the other hand intense sulfate uptake takes place at the osteochondral junction in both mouse and rat ribs and the pictures suggest that the main site for longitudinal enlargement of the rib in growing animals is situated here

The most important consequence of this study seems to be the necessity of a careful selection of cartilage with respect to its anatomical site in metabolic experiments. If the sulfate uptake values into the cartilage are expressed on a weight basis as has been done in many studies the results can be misleading if "active" and "inactive" samples are inadvertently compared

The fact that the high sulfate incorporating activity of the cartilage growth zone is maintained even *in vitro*, indicates that blood circulation is of little importance for the observed activity differences

To avoid mixing of more or less active cartilage pieces Klebanoff (1958) and Yde (1968) selected the samples by discarding the lateral part of the rib cartilage By doing this they have eliminated the metabolically most active part of the tissue The above demonstrated profound regional differences have been subjected to detailed quantitative analysis and the results will be published in a following paper

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References

ALMQVIST S Studies on sulfation factor (SF activity of human serum. Evaluation of an improved method for the in vitro bioassay of SF and the effect of glutamine and human growth hormone in the system *Acta endocr* (Kbh. 1961 *35* 31—50

ANTONOWICZ I and E KODICEK The effect of scurvy on glucosaminoglycans of granulation tissue and costal cartilage *Biochem J* 1968 *110* 609—616

BELANGER, L. F Autoradiographic visualization of the entry and transit of ^{35}S in cartilage, bone and dentine of young rats and the effect of hyaluronidase in vitro *Canad J Biochem* 1954 *32* 161—169

BOSTROM H and E JORPES On the enzymatic exchange of the sulfate group of the animal sulfo-mucopolysaccharides. *Experientia* (Basel 1957 *10* 392—396

BOWNESS J M and M JACOBS Chondroitin sulfate change in puppy rib cartilage during the period of calcification *Canad J Biochem* 1968 *46* 63—67

CAMPO R and D D DZIEWIATKOWSKI Turnover of the organic matrix of cartilage and bone as visualized by autoradiography *J Biophys biochem Cytol* 1963 *18* 19—29

DAUGHADAY W H. and D M KIPNIS The growth promoting and anti insulin action somatropin *Recent Progr Hormone Res* 1966 *22* 49—99

DENKO C W and D M BERGENSTAL Effects of growth hormone and corticosteroids on ^{35}S fixation in cartilage *Endocrinology* 1961 *69* 769—777

ENGFELDT B and O WESTERBORN An autoradiographic study of the epiphyseal cartilage in normal rabbits after administration of radiosulfate *Acta path microbiol scand* 1960 *49* 73—81

FALCONI G and G L ROSSI Transauricular hypophysectomy in rats and mice *Endocrinology* 1964 *74* 301—303

GROSS J I M B MATHEWS and A DORFMAN Sodium chondroitin sulfate protein complexes of cartilage *J biol Chem* 1960 *235* 2889—2892

HOWELL D [illegible] E DELCHAMPS W RIEMER and T KIEM A profile of electrolytes in the cartilaginous plate of growing ribs *J clin Invest* 1960 *39* 919—929

KATSURA N and E A DAVIDSON Metabolism of connective tissue polysaccharides in vivo IV The sulfate group *Biochem biophys Acta* (Amst) 1966 *121* 135—142

KLEBANOFF S J D D DZIEWIATKOWSKI and G J OKINAKA The effect of ascorbic acid oxidation on the incorporation of sulfate by slices of calf costal cartilage *J gen Physiol* 1958 *42* 303—317

KLEINE T O and H HILZ Untersuchungen zur Biosynthese der Chondroitinsulfat Proteine I Charakterisierung der Protein Polysaccharide aus Kalberippenknorpel und ihre In vitro-Markierung mit $^{35}SO_4$ *Hoppe Seylers Z physiol Chem* 1968 *349* 1027—1036

PELC, [illegible] R and A GLUCKSMANN Sulfate metabolism in the cartilage of the trachea pinna and xiphoid process of the adult mouse as indicated by autoradiographs *Exp cell Res* 1955 *8* 336—344

PINKERTON W and M WEBBER A method of injecting small laboratory animals by the ophthalmic plexus route *Proc Soc exp Biol* (N Y) 1964 *116* 959—961

SALMON W D JR and W H DAUGHADAY A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro *J Lab clin Med* 1957 *49* 825—836

SILBERBERG M and R SILBERBERG Steroid hormones and bone In *The biochemistry and physiology of bone* 1956 pp 623—670 Ed G H Bourne New York Academic Press

ULLBERG S Studies on the distribution and fate of ^{35}S labelled benzylpenicillin in the body *Acta radiol* (Stockh.) 1954 Suppl 118 1—110

WHITEHOUSE M W and H BOSTROM Studies on the action of some antiinflammatory agents in inhibiting the biosynthesis of mucopolysaccharide sulfates *Biochem Pharmacol* 1961 *7* 135—150

WHITEHOUSE M W and H BOSTROM Biochemical properties of anti inflammatory drugs — VI The effects of chloroquine (resochin) mepacrine (quinacrine) and some of their potential metabolites on cartilage metabolism and oxidative phosphorylation *Biochem Pharmacol* 1965 *14* 1173—1184

YDE H A simplified technique for the determination of growth hormone dependent sulfation factor using intact animals *Acta endocr* (Kbh) 1968 *57* 557—564

Acta physiol scand 1970. 79 359—368

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Vasoactivity of Adenine Nucleotides Released in vitro from Blood Cells by Thrombin

By

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Abstract

Swedenborg, J *Vasoactivity of adenine nucleotides released from blood cells by thrombin* Acta physiol scand 1970 79 359—368

Small amounts of thrombin infused into the femoral artery of dogs decrease the peripheral vascular resistance due to release of adenine nucleotides from blood cells

The present study was designed to determine the source of vasoactive adenine nucleotides released from blood cells by thrombin Washed human platelets red cells and white cells were incubated with thrombin and the resulting supernatant was tested for vasoactivity Dog platelet rich plasma and platelet poor plasma incubated with thrombin was also assayed for vasoactivity as well as hemolysed red cells Supernatants from incubated platelets showed strong vasoactivity which disappeared after exposure to ADP ase and ATP ase (Apyrase) Supernatants from red cells and white cells were not vasoactive Platelet rich plasma in

It has been shown that thrombin when infused into the femoral artery of the dog in amounts too small to cause formation of intravascular clots gives rise to a marked local decrease in the vascular resistance (Delin Olsson and Teger Nilsson 1967) Less than 0 5 NIH units/ml blood is sufficient to cause significant changes in blood flow The decreased resistance was shown to be due to release of vasoactive material from blood cells by thrombin (Olsson Swedenborg Teger Nilsson 1969 a) It was concluded that the vasodilating material released from blood cells was ADP or ATP or both (Olsson, Swedenborg and Teger Nilsson 1969 b) The type of blood cell from which the main release of the nucleotides occurred was however not determined

The present study was designed to determine the source of the vasoactive nucleotides liberated by thrombin i e whether they originate from red cells, white cells or platelets

Reagents

Adenosine 5' diphosphate (ADP) K & K Chemical Labs Plainview, New York USA
Adenosine 5' triphosphate (ATP) K & K Chemical Labs Plainview, New York USA
Apyrase containing 1.09 U 5'-ATP ase, 0.60 U 5 ADP ase and 0.007 U 5 AMP ase per mg Sigma Chemical Co, St Louis Missouri, USA
Thrombin Parke Davis & Co, Detroit, Michigan, USA
Heparin Sodium (1000 U/ml), Upjohn Kalamazoo, Michigan USA
Wash solution for platelets NaCl 135 mM, disodiumsalt of ethylenediamine tetraacetic acid (EDTA) 1.5 mM, adjusted to pH 7.4 with Tris buffer (tris hydroxymethyl aminomethane)
Concentrated EDTA solution EDTA 50 mM, NaCl 50 mM, pH adjusted to 7.4 with Tris.
Ringer solution NaCl 135 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM and potassium phosphate buffer 4 mM (pH 7.4)

Methods

A Preparation of Washed Blood Cells

Blood (90—100 ml) from healthy male volunteers was drawn into a siliconized bottle containing 1 volume 0.13 M trisodium citrate for every 9 volumes of blood The blood was centrifuged at $150 \times g$ for 20 min The plasma was separated and collected in siliconized tubes The cells were resuspended in the wash solution and centrifuged once more at $150 \times g$ for 20 min The supernatant obtained after this centrifugation was added to the previously obtained plasma in order to collect more platelets The concentrated EDTA solution (see materials) was added 1 ml for every 10 ml of this suspension and it was then cooled to 4° C The platelet suspension was centrifuged at $1200 \times g$ for 20 min at 4° C The supernatant was discarded and the platelet button was washed 3 times in the wash solution and then suspended in Ringer's solution or in the NaCl EDTA solution that had been used for the washings

The cells left after separation of the platelets were resuspended in saline and centrifuged at $1200 \times g$ for 15 min The buffy coat was separated by gentle suction It was possible thereby to get one cell fraction containing most of the white cells and one fraction containing most of the red cells These two cell fractions were washed twice with saline and then suspended in Ringer's solution or in saline All cells were dissolved in a volume equal to one third of the volume of the originally collected blood Samples were taken from the different cell suspensions for counts of white cells red cells and platelets Table I lists the cells c[illegible]nts in the different suspensions

TABLE I Cell Counts in Suspensions Used for Preparation of Supernatants

	Red Cell Count/μl	White Cell Count/μl	Platelet Count/μl
Red Cell Suspensions n = 6	Mean 5.0×10^6 *Range* 4.4×10^6 — 5.6×10^6	Mean 67 *Range* 0—300	Mean [illegible] 500 *Range* 200—31 000
White Cell Suspensions n = 6	Mean 1.4×10^6 Range 0.7×10^6 — 2.2×10^6	Mean 10 800 Range 3 900—18 500	Mean 29 000 Range 12 000—35 000
Platelet Suspensions n = 6	Mean 0 Range 0—0	Mean 270 Range 0—500	Mean 210 000 Range 90 000—280 000

B Preparation of Supernatants

The cell suspensions were incubated at room temperature with different agents and centrifuged thereafter for 15—20 min at 3000 × g. The supernatants were collected and stored at +4° C overnight and tested for vasoactivity the following day. Three different methods were used in obtaining supernatants.

1) Thrombin heparin supernatants. Thrombin was added to the cell suspension which then was gently agitated. After 20 sec heparin was added and thorough mixing was secured. The thrombin concentration was 1 NIH U/ml solvent for red cells, 0.5 NIH U/ml solvent for white cells and 0.1 NIH U/ml solvent for platelet suspensions. Heparin concentration was 10 IU/ml.

2) Heparin thrombin supernatants. Here the order between heparin and thrombin was reversed, but apart from that the supernatants were obtained in exactly the same way.

3) Blank supernatants. Both thrombin and heparin were substituted for saline.

The addition of fluid to the cell suspensions was always less than 0.5 ml/10 ml cell suspension.

One portion of the red cell suspension was hemolyzed by freezing and thawing once. After thawing it was centrifuged at 30 000 × g for 30 min in room temperature. This supernatant was also tested for vasoactivity.

C Preparation of Platelet Rich and Platelet Poor Plasmas

Blood from the experimental animal (100—200 ml) was collected in sodium citrate (1 volume 0.13 M trisodium citrate per 9 volumes of blood). The blood was centrifuged at 150 × g for 20 min and the platelet rich plasma was collected. Approximately half of the platelet rich plasma was spun at 3000 × g for 20 min in order to obtain platelet poor plasma. Samples from both platelet rich and platelet poor plasma were taken for platelet counts. Cells and blood were handled only in siliconized glass or polyethylene material.

D Incubation with Apyrase

When vasoactivity was detected, the supernatants were often incubated with Apyrase to determine whether vasoactivity was due to adenine nucleotides. Incubation was done essentially a[illegible] active supernatant was added 0.5 ml [illegible] r (pH 6.5) and 0.5 ml of 0.03 M [illegible] 0° C. When possible one portion was [illegible]

E Assay of Vasoactivity

Mongrel dogs weighing 12—18 kg were anesthetized with sodium pentobarbital (32 mg/kg b.w.). More pentobarbital was administered when needed. The dogs were assisted with a respirator (Bird Mark 8) during experiments. One femoral artery was exposed and a transducer of a sine wave electromagnetic flowmeter was placed on the artery. The transducer was connected to a Statham Model 4001 flowmeter. Test solutions were injectd through a small polyethylene catheter inserted into a branch of the same femoral artery distally to the flow probe. Injections were made with a Harward infusion pump. Infusion speed was 0.6—4.6 ml/min and the infusions lasted 30—60 sec. Infusions of saline at this speed did not affect blood flow. Since arterial flow and infusion speed were known, it was possible to calculate i.a. concentration for every infusion. Blood pressure was recorded with a Statham strain gauge transducer connected to a polyethylene catheter inserted into one carotid artery. Recordings were made on a Sandborn 350 recorder. During experiments the dogs were heparinized (1000 U/kg b.w.) to avoid any possible direct effects of the thrombin in the supernatants.

Results

Vasoactivity of Supernatants from Washed Blood Cells

A Red Cells

Thrombin heparin supernatants, heparin thrombin supernatants and blank supernatants from the red cell suspensions were tested. All these supernatants showed none or very slight vasoactivity when infused i.a. Blood pressure was not affected. Vasoactivity, when present, was the same in all three types of supernatants. Blood

TABLE II Flow increase by red cell supernatants
Thrombin concentration 1NIH U/ml solvent Heparin concentration 10 U/ml solvent
Mean and range values for 12 i a infusions of each type of supernatant in [illegible] dogs are given
T+H = Supernatant from suspensions where thrombin was added before heparin
H+T = Supernatant from suspensions where heparin was added before thrombin
Blank = Supernatants from suspensions where both heparin and thrombin were replaced by saline

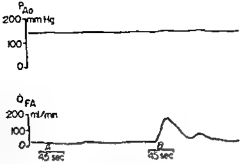
I a concentration µl/ml blood			Flow increase %			Sp act = Flow increase (%) / I a conc (µl ml)		
T+H	H+T	Blank	T+H	H+T	Blank	T+H	H+T	Blank
Mean 62	Mean 59	Mean 61	Mean 32	Mean 35	Mean 29	Mean 0.8	Mean 0.9	Mean 0.7
Range 19—208	Range 18—156	Range 18—179	Range 0—79	Range 0—75	Range 0—100	Range 0—2.6	Range 0—2.[illegible]	Gange 0—2.2

flow and injected amounts were known and thus i a concentrations were calculated Specific activities of the supernatants were expressed as the ratio of flow increase (in per cent) to i a concentration (in µl/ml) of the injected material Resting blood flow was used in the computation of i a concentrations The results from 36 infusions made in 6 dogs are shown in Table II As shown by the table thrombin does not seem to liberate vasoactive material from red cells

In order to estimate the total vasodilating activity in the red cells and the nature of it the supernatants from frozen and thus hemolyzed red cells were tested for vasoactivity These solutions were highly vasodilating when tested on the femoral

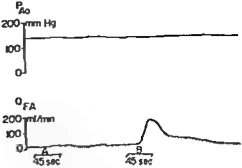
TABLE III Flow increase by supernatants from hemolyzed red cells
I a concentrations 10—312 µl/ml
Mean and range values for 12 infusions before Apyprase and 11 infusions after incubation with Apyrase in 6 dogs are listed

Before Apyrase		After incubation with Apyrase	
Flow increase %	Sp act = Flow increase (%) / I a conc (µl ml)	Flow increase %	Sp act = Flow increase (%) / I a conc (µl ml)
Mean 304	Mean 7.5	Mean 20	Mean 0.2
Range 120—627	Range 1.5—20.2	Range 42—140	Range 0.9—2.6



A = hemolysed red cells incubated with Apyrase
B = hemolysed red cells incubated without Apyrase

Fig 1



A = supernatant incubated with Apyrase
B = supernatant incubated without Apyrase

Fig II

Fig 1 Femoral arterial flow and aortic blood pressure during infusions of supernatants from hemolyzed red cells Infusion speed 2 5 ml/min Infusion time 45 sec
Fig II Femoral arterial blood flow and aortic blood pressure during infusions of supernatants obtained from a suspension of washed human platelets incubated with thrombin (0 1 NIH U/ml) Infusion speed 2 5 ml/min Infusion time 45 sec

artery but did not have any general hemodynamic effects as shown by a constant blood pressure during and after infusions The highly vasoactive hemolysates were incubated with Apyrase After such incubation the hemolysates lost their vasodilating effect Sometimes a slight vasoconstriction was noticed Results from 12 infusions in 6 dogs are listed in Table III Sp act for the supernatants was calculated as described previously Incubation mixtures in which Apyrase was replaced by succinate buffer showed no change in vasoactivity after one hour of incubation A typical experiment is shown in Fig 1

It is concluded that the vasoactivity originating in red cells is due to their content of ADP or ATP or both These nucleotides are liberated at hemolysis but not by thrombin

B White Cells

Suspensions of blood cells with high white cell counts and low red cell counts were used here (Table I) Thrombin heparin and heparin thrombin supernatants were tested 21 infusions were made in 5 dogs Neither thrombin heparin supernatants nor heparin thrombin supernatants showed any vasoactivity It is concluded that thrombin does not release vasoactive material from white cells

C Platelets

The platelet suspensions were incubated with thrombin in a concentration of 0 1 NIH U/ml Thrombin heparin, heparin thrombin and blank supernatants were tested Supernatants prepared with addition of thrombin before the heparin were highly vasoactive Vasoactivity seemed to be the same in supernatants

TABLE IV. Flow increase by platelet supernatants.
Thrombin concentration 0.1 NIH unit/ml solvent. Heparin concentration 10 U/ml solvent.
T+H = Supernatants from suspensions where thrombin was added before heparin.
H+T = Supernatants from suspensions where heparin was added before thrombin.
Blank = Supernatants where both heparin and thrombin were substituted for saline.
Infusions in 6 dogs are listed. In 2 dogs NaCl—EDTA was the suspending medium, in 3 dogs Ringer's and in 1 dog both suspending media were used for the supernatants.

Flow increase %				Sp. act. = $\frac{\text{Flow increase (\%)}}{\text{I.a. conc. } (\mu l/ml)}$			
T+H n = 14	H+T n = 14	Blank n = 14	T+H after incubation with Apyrase n = 10	T+H	H+T	Blank	T+H after incubation with Apyrase
Mean 204	Mean 100	Mean 40	Mean 21	Mean 6.2	Mean 3.0	Mean 1.3	Mean 0.4
Range 72—371	Range 13—226	Range 9—108	Range 0—50	Range 2.1—12.4	Range 0.7—7.5	Range 0.2—4.3	Range 0—1.0

Ringer's as in NaCl EDTA solution. Supernatants to which heparin had been added before thrombin showed some vasoactivity but always less so than the "thrombin heparin supernatants". Supernatants where both thrombin and heparin were replaced by saline showed very slight vasoactivity as compared to the two previously mentioned supernatants. No supernatants had any effect on blood pressure in spite of marked effect locally. For the thrombin heparin supernatants sp. act. was 2.1—12.4 % per µl supernatant/ml blood, mean 6.2. For heparin thrombin supernatants the sp. act. was 0.7—4.5 % per µl/ml, mean 3.0. Blank supernatants showed a sp. act. of 0.2—4.3 % per µl/ml, mean 1.3. Results from 52 infusions in 6 dogs are shown in Table IV.

The vasoactive "thrombin heparin" supernatants were incubated with Apyrase. Vasoactivity disappeared after such incubation (Fig. 2). When Apyrase was replaced by succinate buffer in the incubation mixture no decrease in vasoactivity was seen. The smaller activity of "heparin thrombin supernatants" also disappeared after Apyrase treatment.

ADP and ATP were also infused in every experiment to compare the vasodilating effects to the reaction caused by platelet supernatants. The activity of the supernatants was calculated to correspond to a solution of ADP containing 0.7—3.5 µg/ml or a solution containing 3—9 µg/ml of ATP.

These experiments show that thrombin releases adenine nucleotides with vasodilating properties from platelets. The reaction is blocked only partly by heparin in this test system.

TABLE V *Flow increase by Platelet Rich Plasma (PRP) and Platelet Poor plasma (PPP) incubated with Thrombin. Thrombin concentration was 0.5 NIH U/ml in 2 expts, 0.2 NIH U/ml in 2 expts and 0.1 U/ml in 2 expts. Heparin concentration 10 U/ml*

Flow increase %			Sp act — Flow increased (%) / I a conc (µl ml)			Platelet counts per µl	
Thrombin + Heparin		Heparin + Thrombin	Thrombin + Heparin		Heparin + Thrombin		
PRP n = 13	PPP n = 11	PRP n = 10	PRP	PPP	PRP	PRP	PPP
Mean 170	Mean 32	Mean 10	Mean 6.7	Mean 1.4	Mean 0.5	Mean 370 × 10³	Mean 20 × 10³
Range 71—425	Range 0—95	Range 0—53	Range 1.6—12.9	Range 0—4.8	Range 0—2.4	Range 181 × 10³ 530 × 10³	Range 3 × 10³ 70 × 10³

Vasoactivity of Platlet Rich and Platelet Poor Plasma Incubated with Thrombin

The plasmas were incubated with thrombin in a concentration of 0.1, 0.2 or 0.5 NIH U/ml. Heparin (10 IU/ml) was added after 20 sec, before any clotting was visible and the plasmas were immediately infused. Platelet rich plasma to which heparin had been added before the thrombin and platelet poor plasma without any added material were also tested. Platelet rich plasmas incubated with thrombin gave rise to pronounced vasodilatation. Platelet poor plasmas treated similarly as well as platelet rich plasmas without addition of thrombin gave rise to none or only a small vasodilation. Results from these experiments are shown in Table V. In contrast to the finding with washed platelets heparin is fully capable of preventing the release of vasoactive material from platelets in plasma. The platelet rich plasmas incubated with thrombin had a vasoactivity corresponding to 1—4 µg/ml of ADP or 4—18 µg/ml of ATP.

Discussion

Platelets contain several vasoactive compounds which can be released by thrombin such as serotonin (Rand and Reid 1951, Markwardt and Barthel 1964, Buckingham and Maynert 1964) histamine (Humphrey and Jaques 1954) and adenine nucleotides (Born 1958). The present study indicates that vasoactivity appearing after incubation of platelets with thrombin in vitro is due release of adenine nucleotides. Other platelet constituents seem to be of little or no importance since all activity in the vasocative supernatants disappeared after incubation with Apyrase which is a specific ADP ase and ATP ase with small amounts of AMP ase (Krishnan 1946).

It is reasonable to assume that thrombin induced release of adenine nucleotides occurs also in vivo and thus would explain vasodilation observed when thrombin is injected into the femoral artery (Olsson Swedenborg Teger Nilsson 1969 a) Released ADP or ATP converted to ADP — probably plays a crucial role in platelet aggregation caused by thrombin (Kazer Glansmann and Luscher 1962 Haslam 1964) but along with the aggregation one would also expect a vasomotor effect

The vasoactivity in the supernatants was comparable to that caused by 0 7—3 5 μg/ml of ADP or 3 0—9 0 μg/ml of ATP These figures are slightly higher than similar figures reported earlier (Olsson, Swedenborg and Teger Nilsson 1969 b) but they compare well if the different ways of preparing the supernatants and the relatively rough method of calculation are taken into consideration Since the platelet suspensions had essentially "normal" platelet counts the amounts of ADP or ATP mentioned may well exist in clinical situations where small amounts of thrombin are free to act on the platelets

Results from the experiments where dog plasma was incubated with thrombin further supports the view that appearance of vasoactivity is dependent upon the presence of platelets The activities of platelet rich plasma incubated with thrombin corresponded to 1—4 μg/ml om ADP or 4—18 μg/ml of ATP These figures are slightly higher than the corresponding figures for platelet supernatants The difference may well be explained by the higher platelet counts in the platelet rich plasmas used The concentration of thrombin sometimes had to be increased beyond 0 1 NIH U/ml to demonstrate full effect in dog plasma This may be due to the high anti thrombin capacity in plasma (Seegers Johnson and Fell 1959)

Heparin was not as effective an inhibitor in solutions of washed platelets as it was in plasma The difference in inhibitory effect of heparin in plasma and artificial medium may be due to a presence of platelet factor 4 with antiheparin properties in the artificial medium or to lack of heparin co-factor (Blomback *et al* 1959) Platelets are known to release antiheparin (platelet factor 4) during the process of aggregation (Youssef and Barkhan 1968 Niewiarowsky *et al* 1968) and washing of the platelets might affect them so that antiheparin is readily released The lack of heparin co-factor is a less probable explanation since heparin in this concentration always fully counteracted the thrombin effect in a suspension of washed cells where all three kinds of blood cells were present (Olsson Swedenborg Teger Nilsson 1969 a)

Most of the adenine nucleotide content of blood is to be found in red cells Bell 1935) The nucleotides inside red cells are free to exert their hemodynamic and other possible effects when the cells have been hemolysed The vasodilating effect of red cell hemolysates was shown in this study to be due to the presence of ADP or ATP or both by the fact that the vasoactivity disappeared after exposure to Apyrase Damage to red cells and subsequent liberation of vasoactive material has been reported earlier (Sarajas Kristofferson and Frick 1959 Chambliss *et al* 1960) and the deleterious effects of hemolysis in shock have also been recognized (Hardaway *et al* 1964) Red cells contain a clotting factor which is liberated when hemolysis

occurs (Quick, Georgatsos and Hussey 1954) This has been claimed to be responsible for the clinical effects of hemolysis (McKay 1965), but the local hemodynamic effects of liberated adenine nucleotides as well as their platelet aggregating ability must also be born in mind Nucleotides in red cells, however, seem not to be liberated by thrombin since no vasoactivity exceeding that of the blanks was seen in supernatants from red cell suspensions treated with thrombin

White cells also did not seem to be the source of adenine nucleotides responsible for flow changes caused by thrombin No supernatants from white cells treated with thrombin showed any vasoactivity

Adenine nucleotides are liberated from platelets by small amounts of thrombin When this occurs in vivo under the circumstances of a hyperactive coagulation system and thus in the presence of an increased amount of circulating thrombin, severe changes in vascular resistance may well occur This view is supported by a recent study in which it was found that infusion of thrombin into animals caused an immediate vasodilation before clotting occurred The vascular response seemed to be connected to the platelets (Whitaker and McKay 1969) The amounts of thrombin needed to cause release of vasoactive nucleotides are very small Sufficient amounts could probably be found in most cases with disseminated intravascular coagulation e g in hemorrhagic shock or certain obstetric complications

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References

BLOMBACK B M BLOMBACK B OLSSON G WILLIAM OLSSON and A SENNING Determination of heparin level of the blood *Acta chir scand* 1959 *245* 259—264

BORN G V R Changes in distribution of Phosphorus in platelet rich plasma during clotting *Biochem J* 1958 *68* 695—704

BUCKINGHAM S and E W MAYNERT The release of 5 hydroxytryptamine potassium and amino acids from platelets *J Pharmacol exp Ther* 1964 *143* 332—339

BUELL M V The adenosin nucleotide content of human blood *J biol Chem* 1935 *108* 273—283

CHAMBLISS J R J DEMMING K WELLS W W KLINE and R W ECKSTEIN Hemolysed blood and coronary blood flow *Amer J Physiol* 1950 *153* 545—553

DELIN A B OLSSON and A C TEGER NILSSON Vasodilation in the canine leg caused by intra-arterial infusion of thrombin and tissue thromboplastin *Cardiovascular Research* 1967 *1* 371—378

HARDAWAY R M D G JOHNSON M J ELOVITZ D N HOUCHIN E B JENKINS J W BURNS and H R JACKSON Influence of Trauma and Hemolysis on hemorrhagic shock in dogs *J Trauma* 1964 *4* 624—641

HASLAM R J Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and by fatty acids *Nature* (Lond) 1964 *202* 765—768

HUMPHREY J H and R JAQUES
polymorphonuclear leucocytes of

Row Publishers 1965 23—62 191—207

MARKWARDT, F. and W. BARTHELL. Untersuchungen über die Freisetzung von Serotonin aus Blutplättchen durch Thrombin. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* 1964 *249* 176—184

NIEWIAROWSKI, W., R. LIPINSKI, A. FARBISZEWSKI and A. POPLAWSKI. The release of platelet factor 4 during platelet aggregation and the possible significance of this reaction in hemostasis. *Experientia* (Basel) 1968 *24* 343—344

OLSSON, P., J. SWEDENBORG and A. C. TEGER-NILSSON. Vasodilation and hypotensive effect of thrombin in dogs. Role of Fibrinogen plasma kinins and formed elements of the blood. *Cardiovascular Research* 1969 a *3* 56—63

OLSSON, P., J. SWEDENBORG and A. C. TEGER-NILSSON. The role of adenine nucleotides in the peripheral vasodilating effect of thrombin. *Acta physiol. scand.* 1969 b *76* 137—147

QUICK, A. J., J. G. GEORGATSOS and C. V. HUSSEY. The clotting activity of human erythrocytes. Theoretical and clinical implications. *Amer. J. med. Sci.* 1954 *228* 207—213

RAND, M. and G. REID. Sources of serotonin in serum. *Nature* (Lond.) 1951 *168* 385

SARAJAS, H. S. S., R. KRISTOFFERSSON and M. H. FRICK. Release of adenosine triphosphate and serotonin from injured cellular blood elements in extracorporeal circuits. *Nature* (Lond.) 1959 *184* 1127—1128

SEEGERS, W. H., J. F. [illegible] activation. *Amer.* [illegible]

WHITTAKER, A. N. and [illegible] by intravenous thrombin infusion. *Lab. Invest.* 19[illegible] *20* [illegible]

YOUSSEF, A. and P. BARKHAN. Release of platelet factor 4 by adenosine diphosphate and other platelet aggregation agents. *Brit. med. J.* 1968 *1* 746—747

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Myoneural Ultrastructure and Cholinesterases after Tetrodotoxin Treatment

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Abstract

TERÄVÄINEN, H. *Myoneural ultrastructure and cholinesterases after tetrodotoxin treatment.* Acta physiol. scand. 1970. 79. 369—372.

Intraperitoneally injected tetrodotoxin, irrespective of the dose given, caused no changes in the ultrastructure of the myoneural junctions of the rat striated muscle or in their histochemically demonstrable acetyl (E.C. 3.1.1.7.) or non-specific (E.C. 3.1.1.8.) cholinesterase activity.

Tetrodotoxin is an extremely toxic non-protein substance quite lately discovered to cause total block of neuromuscular transmission in a very short time by affecting conduction in both the pre- and postsynaptic membranes. Electrophysiological examination of the effect of tetrodotoxin on synaptic transmission has only been begun during the last few years and the results obtained have lately been reviewed by Kao (1966). The exact mechanism by which this drug exerts its effect is unknown, but it has been shown to abolish impulse conduction through the myoneural junction by selectively blocking the transfer of sodium through the depolarized membrane (Narahashi *et al.* 1960, Narahashi *et al.* 1964, Nakamura *et al.* 1965). It does not interfere with the release of acetylcholine from the nerve ending (Elmqvist and Feldman 1965, Katz and Miledi 1967, Miledi 1967) nor does it prevent the depolarizing action of acetylcholine in the myoneural junction (Furukawa *et al.* 1959). Tetrodotoxin is believed slightly to reduce the total cholinesterase activity of the myoneural junction (*cf.* Fleisher *et al.* 1961, Dettbarn *et al.* 1965, Kao and Nishiyama 1965).

The aim of the present investigation was to see whether tetrodotoxin produces any change in the ultrastructure or the acetylcholinesterase (E.C. 3.1.1.7.) or other cholinesterase (non-specific, E.C. 3.1.1.8.) activity of the myoneural junction, in view of the fact that the non-specific cholinesterase activity in different tissues has been suggested to be related to ion transport (*e.g.* Kamemoto 1961, Hertz 1968, Fourman 1969, *cf.* also Florey 1965).

Material and Methods

A commercially available tetrodotoxin preparation buffered with citric acid and sodium citrate (Tetrodotoxin, Crystalline 3X, Sankyo Co, Ltd Tokyo Japan) was dissolved in water to give a stock solution containing a final concentration of 200 μg tetrodotoxin in 1 ml The tetrodotoxin solution was injected i p in doses ranging from 4 μg/kg (sublethal) to lethal doses between 12 μg/kg and 100 μg/kg in to a total of 14 Sprague Dawley rats 2 months old. Three untreated rats were used as controls and killed by a blow on the head at the moment when the animals that had received tetrodotoxin stopped breathing After a lethal dose the animals lived only a few minutes

The intercostalis muscle [illegible] lar contractions ceased fixed for electron microsc [illegible] M phospate at pH 7 2 (Sabatini *et al* 1963) for [illegible] O_4 for 1 5 hrs in 0 1 M phosphate buffer at pH [illegible] h graded ethyl alcohol series embedded in Epon 812 (Luft 1961), sectioned, and stained on grids with lead citrate (Reynolds 1963)

Histochemical tests for myoneural cholinesterase activity were made from the anterior tibial muscles which were used either unfixed or fixed at 4° C for 8 hrs in a solution containing 3 5 % neutralized formaldehyde and 1 % $CaCl_2$ The frozen muscles were sectioned at 20 μ and free floating sections were used throughout the histochemical experiments Total cholinesterase activity and acetylcholinesterase were determined with acetylcholine iodide (Fluka A G Buchs) as substrate and other cholinesterase activity with butyrylthiocholine iodide (Fluka A G Buchs) as substrate When AChE was demonstrated 10^{-6} M tetra isopropyl pyrophosphoramide (iso-OMPA L Light & Co Ltd Colnbrook) was used in the incubation solution to exclude hydrolysis of acetylthiocholine iodide by ns ChE To demonstrate ns ChE AChE was inhibited with 10^{-5} M 284C51 (1 5 bis (4 allyl dimethyl ammoniumphenyl)pentan 3-one diiodide Wellcome Research Laboratories) The effect of both inhibitors was tested using both iso OMPA and 284C51 in the incubation solution with acetylthiocholine as substrate the results were negative The sections were always preincubated for 30 min without any substrate and then incubated with the appropriate substrate for 60 min 120 min and 180 min in both controls and experiments In order to demonstrate the expected irreversibility (Haleman *et al* 1969) of the tetrodotoxin effect tests were also made for myoneural cholinesterases in sections exposed to 40 μg tetrodotoxin in 10 ml of the histochemical incubation solution This amount greatly (by about 330 times) exceeds the lethal dose for the rat reported by the manufacturer to be 12 μg/kg injected i p The intensity of the histochemical reaction was estimated visually

Results

Irrespective of the dose given and the length of survival of the animal the fine structure of the myoneural junction after tetrodotoxin injection was in all essentials the same as that of the control muscles The number of synaptic vesicles as well their morphology appeared to be normal No structural changes were observed in the neurofilaments neurotubules or mitochondria of the axon terminal these being in no way different in morphology from those of the controls in the present study or from those of normal myoneural junctions earlier described (Robertson 1960 Andersson Cedergren 1959 Teravainen 1968 and others *cf* Zacks 1964) The postsynaptic part of the myoneural junction including the postsynaptic membrane and its infoldings sarcoplasm mitochondria and sarcoplasmic tubules was likewise unaltered by tetrodotoxin treatment

Histochemical tests revealed intense AChE activity in the myoneural junction both in normal and in tetrodotoxin treated animals whether or not tetrodotoxin was added to the incubation solution Quantitative estimations revealed AChE reactions of similar intensity in both the controls and the experimental animals after all incubation times The distribution of the cholinesterase activity revealed no histo-

chemical alterations in the myoneural junction with light microscopy In contrast to the myoneural AChE activity, myoneural ns ChE activity was also present in the teloglial cells and the intensity of the ns ChE reaction in the normal junctions was comparable with that of the tetrodotoxin treated animals whether tetrodotoxin was present in the incubation solution or not Neither were structural alterations revealed by the histochemical distribution of the ns ChE activity in tetrodotoxin treated animals compared with the controls

Discussion

The author is aware that histochemical determination of cholinesterase activity is not an accurate method for quantitative estimation However, when the estimation is made using the same incubation and fixation solutions temperatures and times as well as different incubation times with and without fixation for estimation of the reaction intensity relatively precise discrimination can be achieved No differences in activity were observed between the controls and the tetrodotoxin treated animals which indicates that tetrodotoxin does not reduce myoneural AChE or ns ChE activity, at least to any great extent

Unlike the various cholinesterase inhibitors (Preusser 1967 Fischer 1968 Ariens *et al* 1969) the injected tetrodotoxin did not cause structural alterations in the pre or postsynaptic parts of the myoneural junction whether estimated from the structure revealed by the histochemically demonstrated distribution of AChE or ns ChE activity or by electron microscopy This absence of structural alterations does not contribute towards explaining the basic action of tetrodotoxin on cholinergic synapses The observation that tetrodotoxin does not cause reduction of myoneural cholinesterase activity rules of the possibility that tetrodotoxin specifically interferes with sodium conductance by affecting myoneural cholinesterase activity It is interesting therefore to note that neither does tetrodotoxin block sodium conductance through frog skin or in the proximal tubule of the kidney (Solomon 1969)

References

ANDERSON CEDERGREN E Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fibre as revealed by three-dimensional reconstructions from serial sections *J Ultrastruct Res* 1959 2 Suppl 1 1—191

ARIENS A TH E MEETER O L WOLTHUIS and R M J VAN BENTHEM Reversible necrosis at the end plate region in striated muscles of the rat poisoned with cholinesterase inhibitors *Experientia* (Basel) 1969 *25* 57—59

DETTBARN W D H B HIGMAN E BARTELS and T PODLESKI Effects of marine toxins on electrical activity and K efflux on excitable membranes *Biochem biophys Acta* 1965 *94* 472—478

ELMQVIST D and D S FELDMAN Spontaneous activity at a mammalian neuromuscular junction in tetrodotoxin *Acta physiol scand* 1965 *64* 475—476

FISCHER G Inhibierung and Restitution der Azetylcholinesterase an der motorischen Endplatte im Zwerchfell der Ratte nach Intoxikation mit Soman *Histochemie* 1968 *16* 144—149

FLEISHER I H P I KILLOS and C S HARRISON Effects of puffer poison on neuromuscular transmission *J Pharmacol* 1961 *133* 98—105

FLOREY, E, Comparative pharmacology, Neurotropic and myotropic substances *Ann Rev Pharmacol* 1965 *5* 357—382

FOURMAN, J, Cholinesterase activity in the supra-orbital salt secreting gland of the duck *J Anat* 1969 *104* 233—239

FURUKAWA T, T SASAOKA and Y HOSYA Effects of tetrodotoxin on the neuromuscular junction *Jap J Physiol* 1959 *9* 143—152

HAFEMANN D R, A COSTIN and T J TARBY, Neurophysiological effects of tetrodotoxin in lateral geniculate body and dorsal hippocampus *Brain Res* 1969 *12* 363—373

HERTZ L Potassium effects on ion transport in brain slices *J Neurochem* 1968 *15* 1—16

KAO C Y and A NISHIYAMA Actions of saxitoxin on peripheral neuromuscular systems *J Physiol* (Lond) 1965 *180* 50—66

KAO, C Y, Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena *Pharmacol Rev* 1966 *18* 997—1049

KAMEMOTO F I The effects of eserine on sodium regulation in crayfish *Comp Biochem Physiol* 1961 *3* 297—303

KATZ B and R MILEDI Tetrodotoxin and neuromuscular transmission *Proc R Soc B* 1967 *167* 8—22

KOELLE G B, The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions *J Pharmacol exp Ther* 1951 *103* 153—171

LUFT, J H, Improvements in epoxy resin embedding methods *J biophys biochem Cytol* 1961 *9* 409—414

MILEDI R Spontaneous synaptic potentials and quantal release of transmitter in the stellate ganglion of the squid *J Physiol* (Lond) 1967 *192* 379—406

NAKAMURA, Y, S NAKAJIMA and H GRUNDFEST, The action of tetrodotoxin on electrogenic components of squid giant axons *J gen Physiol* 1965 *48* 985—996

NARAHASHI T T DEGUCHI N I [illegible] muscle fibre membrane by tetro[illegible]

NARAHASHI T J W MOORE and [illegible] increase in lobster giant axons [illegible]

PREUSSER H J Die Ultrastrukt[illegible] Veranderungen nach Inhibieru[illegible] 457

REYNOLDS E S The use of lead citrate at high pH as an electron opaque stain in electron microscopy *J cell Biol* 1963 *17* 208—212

ROBERTSON J D Electron microscopy of the motor end plate and the neuromuscular spindle *Amer J phys Med* 1960 *39* 1—43

SABATINI D D K BENSCH and R J BARRNETT Cytochemistry and electron microscopy The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation *J cell Biol* 1963 *17* 19—58

SOLOMON S Failure of tetrodotoxin to affect sodium transport by frog skin and proximal tubule of rat kidney *Life Sci* 1969 *8* 397—400

TERÄVÄINEN H Development of the myoneural junction in the rat *Z Zellforsch* 1968 *87* 249—265

ZACKS S I *The motor end plate* Philadelphia and London Saunders 1964

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Structurally Based Increase of Flow Resistance in Spontaneously Hypertensive Rats

By

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Abstract

Folkow, B M Hallbäck, Y Lundgren and L Weiss *Structurally based increase of flow resistance in spontaneously hypertensive rats* Acta physiol scand 1970 79 373—378

The resistance to flow in the maximally dilated systemic vascular bed (except the coronaries) was compared in spontaneously hypertensive rats (SHR) and normotensive controls — The aortic root was cannulated for perfusion with an oxygenated plasma substitute both the aortic inflow and the venous effluent via the right heart being recorded continuously Temperature and viscosity of the perfusate were kept constant and it was checked with supramaximal doses of vasodilator drugs that complete vascular relaxation was at hand Pressure flow curves were plotted and flow resistances at equal pressure heads and transmural pressures were compared — Flow resistance in the maximally dilated systemic vessels was increased in the spontaneously hypertensive rats to an extent that largely equalled their raised blood pressure during "resting" conditions, the difference to the control animals being highly significant — The results suggest the presence of a morphological adaptation of the resistance vessels of the entire systemic circuit which raises the structurally set "baseline for functional vascular adjustments and which therefore may largely account for the increased flow resistance during rest These findings are in agreement with earlier studies concerning the regional flow resistance in hypertensive man (Folkow 1956 Sivertsson and Olander 1968)

The exact background of the raised systemic resistance in the most common type of high blood pressure in man essential hypertension is not known despite much effort The wellknown fact that the resistance vessels of such patients dilate readily when exposed to vasodilator influences has clearly shown that the high resistance is not a matter of any sclerotic vascular narrowing Therefore most investigators have directed their interest to the activity level of the vascular smooth muscles and at present the involvement of different combinations of blood borne and neurogenic excitatory influences are much debated

It is quite possible however, that a type of structural vascular change other than wall sclerotization may be involved For instance adaptive vascular changes in the form of a hypertrophic rebuild of the wall may take place perhaps triggered more or less intermittent

rebuild may reset to a higher level a *per se* fully normal range of vasoconstriction vasodilatation. It may also have the consequence that greater increases of resistance are produced by a given shortening of the smooth muscles, because the wall mass situated inside the "line of force" for muscle contraction would be increased (Folkow 1956).

In fact, it has been shown that even at maximal vasodilatation the flow resistance in the forearm of subjects with essential hypertension is raised largely in proportion to their resting arterial pressure (Folkow 1956, Folkow, Grimby and Thulesius 1958). This does, of course, by no means deny the fact that vascular smooth muscle activity, compared with normotensive subjects, can frequently be much enhanced in an intermittent fashion in patients with essential hypertension. The findings suggest, however, that their systemic resistance vessels — rather than exhibiting a continued increase of smooth muscle activity as the sole, or even main background of the raised pressure — have developed an adaptive thickening of their walls or other type of rebuild, that has encroached to some extent on the lumen.

It was further recently shown on the hand blood vessels of hypertensive patients by other members of the same group (Sivertsson and Olander 1968) that standardized excitory influences produce exaggerated vascular responses of such a nature as to suggest the presence of a wall thickening rather than any increased smooth muscle sensitivity or "reactivity". It has also been shown (Folkow and Sivertsson 1968) that morphological vascular changes of the mentioned adaptive nature appear to be established already within few weeks after a changed pressure load, in other words it is a fairly rapid process and not any late "complication".

All the mentioned studies were, however, confined to isolated vascular beds and the present experiments are intended to explore whether changes of this nature are more or less generalized to the entire systemic circulation. A preliminary report of the present study was recently published (Folkow *et al.* 1969).

Material and methods

The experiments were performed on 12 spontaneously hypertensive rats (SHR) of the Wistar strain (Okamoto 1963) of both sexes, aged 7 months or more, and 12 normotensive controls (NCR) of the Wistar or Sprague Dawley strains of corresponding age.

The animals were anesthetized with nembutal 3 mg/100 g b.w. i.p. and the trachea was cannulated to permit a free airway. The animals were heparinized and arterial pressure was measured via a polyethylene tube in the femoral artery. The tube was connected to a Statham pressure transducer, recording on a Grass Polygraph Model 7. While this recording was performed, guanethidine 0.5–1 mg/100 g b.w. was given in order to block the sympathetic nervous system. In addition it was tested whether isopropylnoradrenaline and acetylcholine were suited to produce maximal dilatation of the systemic vessels during the perfusion part of the experiment by exploring their depressor effects when injected intravenously.

The thorax was then opened over the heart while the rats were artificially ventilated by a Palmer AC respiration pump, the chest muscles being divided between ligatures. A cannula connected to a container with the perfusate was introduced into the aortic root so that the entire systemic vascular bed except the coronaries could be perfused. A wide bore tube was inserted through the wall of the right ventricle with the tip in the right auricle, so that it drained the entire venous outflow from the systemic circuits. The outflow end of the tube was placed at the level of the heart. Flow was recorded both on the "arterial" side by a Medicon Model 2000 Microflow electromagnetic flow meter and on the venous side by collecting the outflow in a graded test tube. The perfusate consisted of oxygenated Tyrode solution to which

4 % Ficoll (a synthetic polymer of sucrose and epichlorohydrin, m w around 80,000, kindly supplied by AB Pharmacia, Sweden) was added to maintain a reasonably normal colloid osmotic pressure in the perfusate The viscosity of the perfusate was in each experiment determined in a Wells Brookfield Micro viscosimeter

The perfusion pressure was altered by changing the level of the container with the perfusate, mostly the lower pressure range was utilized to avoid oedema formation and to ensure [illegible] adrenaline was given at the end of the experiment which always resulted in a constriction Pressure flow curves for the maximally dilated vessels were then constructed from repeated flow measurements at different pressures

Results

Mean arterial blood pressure of the two grups of anesthetized rats showed a significant difference, being 123 ± 4 mm Hg in the normotensive control rats (NCR) and 174 ± 9 mm Hg in the spontaneously hypertensiv rats (SHR) Guanethidine was given intravenously *in order to block sympathetic cardiovascular control and iso*propylnoradrenaline was then injected in huge amounts (up to 10 μg or more) to produce a transitory and largely maximal systemic vasodilatation After guanethidine the pressure fell in the normotensive rats to a mean value of 59 ± 9 mm Hg and in the SHR to a mean value of 64 ± 3 mm Hg and after a supramaximal dose of isopropylnoradrenaline to 36 ± 3 mm Hg in the NCR and to 48 ± 3 mm Hg in the SHR.

During the subsequent perfusion with the plasma substitute the perfusion pressure was altered between zero and 40 mm Hg, higher pressures being only exceptionally used in order to avoid oedema formation The flow was measured repeatedly at each pressure level during steady state conditions and was calculated per 100 g of tissue Repeated injections of huge amounts of isopropylnoradrenaline and acetylcholine indicated that the vessels were maximally dilated as no increase of flow was ever seen upon the vasodilator drug administration Pressure-flow curves were then plotted for each experiments and the mean values with S E M for each group are shown in Fig 1 Computing the flow resistance in PRU_{100} (mm Hg/flow in ml/min $\times$ 100 g

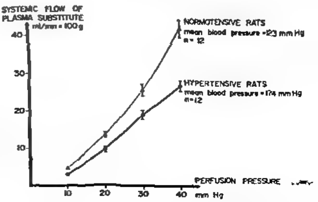

Fig 1 Pressure flow curves showing mean values of 24 technically entirely successful experments on 12 normotensive and 12 hypertensive rats Vertical bars indicate the standard error of the mean The two curves are significantly separated ($p < 0.001$)

tissue) at equal levels of perfusion pressure and transmural pressure, the hypertensive rats had a significantly higher systemic flow resistance at complete vascular relaxation than the normotensives ($p<0.001$). It was in the range of a 50 % increase which in size corresponded well with the difference in "resting" blood pressure during standardized anesthesia (Fig. 1). No significant difference was in this respect observed between the normotensive Wistar and Sprague Dawley control rats.

Discussion

In essential hypertension in man, flow resistance in the forearm is raised largely in proportion to resting arterial pressure, even when the vascular smooth muscles are completely relaxed (Folkow 1956, Folkow, Grimby and Thulesius 1958, Conway 1963). Moreover, it can be predicted that the presence of an adaptive structural increase of wall/lumen ratio of the resistance vessels would result in exaggerated resistance increases for a given active shortening of the vascular smooth muscles thanks to the increased wall mass situated inside the line of smooth muscle shortening (Folkow 1956). In agreement with these theoretical predictions the responses of the hand blood vessels to increasing noradrenaline amounts suggest the presence of structural increase of wall/lumen ratio in hypertensive subjects when compared with matched normotensive controls (Sivertsson and Olander 1968). Thus the threshold to noradrenaline is about the same in the two groups while suprathreshold doses produce increasingly exaggerated responses in the hypertensive subjects.

Such functional results are in agreement with morphological studies indicating that a generalized wall thickening of the arterioles is present in chronic hypertension (see Pickering 1968), also occurring within the pulmonary vascular bed when blood pressure is increased in this circuit (Ferguson and Varco 1955). The reverse seems to occur as expected in prolonged hypotension. Folkow and Sivertsson (1968) observed a reduction in wall/lumen ratio of the arteries in an artificially hypotensive limb of a normotensive cat. Further, the vessels of the chronically hypotensive limb showed decreased responses to noradrenaline in comparison with the normotensive limb, while the vascular sensitivity in terms of the threshold to noradrenaline was largely the same in the two limbs.

In fact adapative hypertrophy appears to occur in virtually all tissues exposed to increased "strain", especially in mesodermal tissues (see e.g. Folkow and Sivertsson 1968). Thus it has been suggested that such changes might account also for the "resetting" of the arterial baroreceptors simply by making the vascular walls at the site of the receptors less distenable, which has been shown to be the case in recent experiments on rabbits (Aars 1969).

In the present study of the systemic vascular bed, with exception of the coronaries (which, however, receive at most some 5 % of the flow when the systemic vascular bed is maximally dilated), it was found that the resistance of the completely relaxed vessels was raised in the spontaneously hypertensive rats to about the same extent as was their arterial blood pressure during standardized "resting" conditions. It follows

hat the raised flow resistance in these animals may be almost entirely accounted for y a structural change of the resistance vessels resetting the baseline for dynamic ascular control to a higher level and hence permitting a quite normal level of vascular smooth muscle activity during the resting hypertensive equilibrium This does of course by no means deny that increased smooth muscle activity may be present intermittently *e g* as a result of increased sympathetic activity perhaps hen acting as a trigger for a morphological adaptation of the vessels

In several respects the type of raised blood pressure which occurs in the spontaneously hypertensive rats (Okamoto and Aoki 1963, Okamoto *et al* 1966) utilized in his study, may be considered the best available animal model of essential hypertension in man The present results indicate that these animals exhibit more or less hroughout their systemic vascular bed a similar increase of the structurally determined flow resistance as has been shown to be present in regional circuits of subjects with essential hypertension It may then be questioned first how rapidly can a morphological vascular change of this type develop and second may it represent the genetically linked element in the hypertensive state?

As to the first question it has been shown that vascular adaptive changes are fully evident in the hindlimb of cats within 3 weeks after a change of the pressure load perhaps earlier (Folkow and Sivertsson 1968) In the aorta at the site of the baroreceptors such changes can be traced even within a week or so (Aars 1969) Thus even if a structural change of this type may well represent a secondary response to some functional trigger factor it seems to develop so rapidly that it would be closely intermingled with the hypothetical trigger" mechanism whenever this exerts a reasonably prolonged or often repeated action

As to the second question it is *a priori* quite possible that the genetically linked factor is constituted by some type of functional trigger mechanism such as an abnormally enhanced sympathetic impact on the cardiovascular system etc However the genetically linked element may also be constituted by an especially pronounced tendency of the blood vessels to respond with hypertrophic wall changes when exposed to a *per se* fully normal extent of intermittently increased pressure load in connection *e g* with such emotionally charged stimuli that belong to a normal environment (see Folkow and Rubinstein 1966) If this latter alternative is the correct one such a cardiovascular system would be prone to gradually develop hypertension even when there is no primary disturbance of the functional equilibrium beyond what is normally encountered by all organisms Further studies are necessary to elucidate this problem

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References

AARS, H. Effects of arterial hypertension on aortic baroreceptor activity. *J. of Oslo Cy Hosp.* 1969.

CONWAY, J. A vascular abnormality in hypertension: a study of blood flow in the forearm. *Circulation* 1969. 27. 520.

FERGUSON, D. J. and R. L. VARCO. The relation of blood pressure and flow to the development and regression of experimentally induced pulmonary arteriosclerosis. *Circulat. Res.* 1955. 3. 152—158.

FOLKOW, B. Structural, myogenic, humoral and nervous factors controlling peripheral resistance. *Hypotensive Drugs.* 1956. Pergamon Press, London.

FOLKOW, B., G. GRIMBY and O. THULESIUS. Adaptive structural changes of the vascular walls in hypertension and their relation to the control of the peripheral resistance. *Acta physiol. scand.* 1958. 44. 255—271.

FOLKOW, B., M. HALLBÄCK, Y. LUNDGREN and L. WEISS. Structurally based increase of flow resistance in spontaneously hypertensive rats. *Acta physiol. scand.* 1969. Suppl. 330. 94.

FOLKOW, B. and E. H. RUBINSTEIN. Cardiovascular effects of acute and chronic stimulation of the hypothalamic defence area in the rat. *Acta physiol. scand.* 1966. 68. 48—57.

FOLKOW, B. and R. SIVERTSSON. Adaptive changes in reactivity and wall/lumen ratio in rat blood vessels exposed to prolonged transmural pressure difference. *Life Sci.* 1968. 7. I. 1283—1289.

OKAMOTO, K. and K. AOKI. Development of a strain of spontaneously hypertensive rats. *Jap. Circulat. J.* (En.) 1963. 27. 282—293.

OKAMOTO, K., R. TABEI, M. FUKUSHIMA, S. NOSAKA, Y. YAMORI, K. ICHIJIMA, H. HAEBARA, M. MATSUMOTO, T. MARUYAMA, Y. SUZUKI and M. TAMEGAI. Further observations on the development of a strain of spontaneously hypertensive rats. *Jap. Circulat. J.* (En.) 1966. 30. 703—716.

PICKERING, G. *High blood pressure.* Ed. 2. J. & A. Churchill Ltd. London. 1968.

SIVERTSSON, R. and R. OLANDER. Aspects of the nature of the increased vascular resistance and increased reactivity to noradrenaline in hypertensive subjects. *Life Sci.* 1968. 7. I. 1291—1297.

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Characterization of Ovarian RNA

Extraction, fractionation by agarose-polyacrylamide gel electrophoresis, and labelling pattern of RNA species in the prepubertal rat ovary

By

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Abstract

AHREN, K., L. HAMBERGER, J. JARLSTEDT and L. NILSSON *Characterization of ovarian RNA Extraction, fractionation by agarose-polyacrylamide gel electrophoresis and labelling pattern of RNA species in the prepubertal rat ovary* Acta physiol scand 1970 79 379—389

[illegible]

shorter incubation periods with most of the radioactivity concentrated to RNA fractions of molecular weights higher than 28S, but also clear labelling of the 5S—4S region After 60 min radioactivity peaks were also associated with the 28S and 18S fractions and with fractions between those while after 240 min incubation the radioactivity was concentrated to the four main fractions The incorporation of radioactivity was found to be markedly decreased in presence of actinomycin D

The mammalian ovary is a complex organ and many hormonal factors of both pituitary and placental origins are engaged in its regulation of growth, development and function Present knowledge concerning the mechanism of action of these gonadotrophic factors is however still limited Some progress, however has been made within this field of research during recent years It has for example been reported that gonadotrophins can rapidly stimulate different enzyme reactions in-

Abbreviations AMPS, amoniumperoxodisulphate, Bis, acrylamide-N, N'-methylenebisacryl amide DPM disintegrations/min, KAc, potassium acetate, PPO 2, 5-diphenyloxazole POPOP 1, 4 bis-(5 phenyloxazolyl-2)-benzene, SDS, sodium dodecyl sulphate, TEMED N, N, N, N' tetramethyl-ethylenediamine

volved in ovarian steroidogenesis (Savard, Marsh and Rice 1965), but it is not known whether this is due to an activation of pre-existing enzymes or to the formation of new enzyme protein. Acute effects of gonadotrophins on amino acid transport and incorporation of labelled amino acid into ovarian protein as well as on various steps of the ovarian carbohydrate metabolism have also been reported (Ahrén, Hamberger and Rubinstein 1969).

Very few experiments have, however, heretofore been performed with the aim of studying the metabolism of ovarian ribonucleic acids. Histochemical studies have suggested that gonadotrophins stimulate the general synthesis of nucleic acids (Jacobs 1962), and an increase in total RNA extracted by the method described by Schneider (1957) and determined by the orcinol assay (Drury 1948) has been observed in the prepubertal rat ovary after treatment with gonadotrophins (van Dyke and Katzman 1968). An increase in incorporation of labelled uridine into RNA of rabbit ovarian slices *in vitro* after pretreatment *in vivo* with gonadotrophins has also been reported (Civen, Brown and Hilliard 1966). In the last mentioned study, the incorporation of uridine was determined in ribonucleotides after treatment of the labelled RNA with pancreatic ribonuclease. The same type of method for the determination of precursor incorporation into RNA was used in recently published investigations on prepubertal rat ovaries (Reel and Gorski 1968 a, b). We think, however, that a further analysis of the gonadotrophic regulation of precursor incorporation into *total* ovarian RNA or into only crudely defined fractions of RNA is of limited value.

No studies have up to now been reported on the fractionation and separation of various RNA species from the ovary, and thus the labelling pattern of various RNA species is also unknown. The present project was started in order to analyze the various species of ovarian RNA, and to study effects of gonadotrophins on the synthesis and labelling pattern of these fractions. The present publication describes an extraction method and fractionation of RNA from prepubertal rat ovaries by agarose polyacrylamide gel electrophoresis. Incorporation of labelled uridine added *in vitro* to the isolated whole ovaries will also be described. The prepubertal rat ovary was chosen for these studies since it has been shown that this type of ovary provides an excellent system for studying the mechanism of action of gonadotrophic hormones *in vitro* (Ahrén *et al.* 1969).

Material and methods

Animals

Rats of the Sprague Dawley strain, supplied by Anticimex Ltd, Stockholm, Sweden, were used. The rats were kept in a room with a constant temperature of 25° C for 4 days, providing alternatively 14 hrs of light and 10 hrs of darkness, and maintained on a semi-synthetic diet (Gustafsson 1959) and tap water *ad libitum*. The rats were used for experiments when 24—26 days old. 20—24 hrs before the onset of the experiments all rats were deprived of food.

Removal and incubation of ovaries

The rats were killed by cervical fracture. The ovaries were rapidly removed, dissected free from bursae and extraneous tissue under a *Zeiss* stereomicroscope and placed in ice-cold Krebs-Ringer bicarbonate buffer. Ovaries from 10 rats were pooled, representing a wet weight of 100—120 mg. Before the start of the incubation, the ovaries were gently blotted on filter paper and

they were then transferred to new flasks with medium containing both actinomycin-D and the isotope Following incubation the ovaries were immediately removed from the flasks, blotted on filter paper and frozen on dry ice

RNA extraction

interphase was transferred to new tubes, to which an equal volume of a mixture of buffer A and phenol (1 1) was added The extraction was repeated once, as mentioned above, with the interphase material, whereafter the water phases were pooled Phenol was then added to the water phase (1 1) and shaken on a supermixer for 5 min at room temperature The sample

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were
The

ether was suctioned away and this last procedure was repeated twice RNA was precipitated with two volumes of a mixture of 95 % ethanol and ether (3 1) and was stored overnight at $-20°$ C The precipitate was centrifuged at 5—6000 rpm for 30 min at 4° C, the ethanol-ether was carefully removed and the precipitate dissolved in 0 02 M Tris HCl, pH 8 1, containing 0 02 M NaCl and 0 002 M EDTA (buffer B) The RNA solution was precipitated in two volumes of 95 % ethanol and stored for at least 1 hr at $-20°$ C the precipitate centrifuged and redissolved in smallest possible amount of buffer B The concentration of RNA in the samples was determined by the absorbance at 260 mμ, read in a Zeiss spectrophotometer, PM Q II As a test of the purity of the preparations, an absorbance curve ranging from 220 mμ—280 mμ was made In all samples there was a sharp peak at 260 mμ with low absorbance at 230 and 280 mμ The 260 mμ/280 mμ ratio was regularly 2 1 (2 05—2 15) In some experiments the samples were treated with DNase The precipitate was dissolved in 0 01 M Tris buffer

dure was used In other experiments pronase in a final concentration of 0 5 μg/ml, was added directly to the extraction medium (buffer A)

Agarose polyacrylamide electrophoresis

Apparatus

A rectangular electrophoresis tank was made of perspex according to Wieme (1965) with 2 % agarose blocks moulded in separate chambers in both ends, leaving a free space in the middle of the tank, appropriate for the gel plate Buffer B was poured upon the agarose blocks,

Chemicals

Agarose was purchased from L'Industrie Biologique Française. Bis and TEMED were obtained from Eastman Organic Chemicals and AMPS from Merck AG. Stock solutions of 1 % Bis and 15 % acrylamide were made (these solutions have limited durability and must be used within a week), and 10 % AMPS. Buffer B was used as dissolving agent. All solutions were kept under refrigeration until immediately prior to the gel preparation.

Gel preparation and electrophoresis

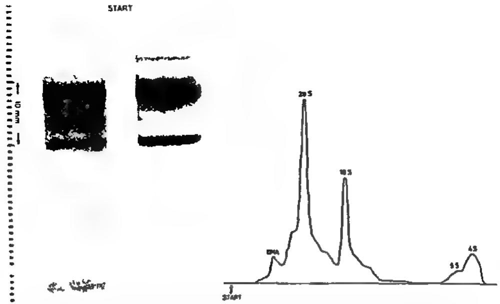
Gels were prepared according to Peacock and Dingman (1968) with certain modifications. 0.075 g agarose was dissolved in 11.5 ml buffer B in an Erlenmeyer flask which was kept in boiling water for 10 min. The clear agarose solution was cooled to 30—35° C with tap water. To the Erlenmeyer flask was now added 1.5 ml Bis, 2 ml acrylamide, 100 µl AMPS and 10 µl TEMED, yielding a composite gel with 0.5 % agarose and 2 % polyacrylamide. This was done at room temperature and the flask was shaken after adding each of the constituents. The solution was now poured into the gel form and the gel polymerized after about 20 min. The gel was ... tank in the cold ... blocks with newly ... sed to stabilize for ... ptimum concentra ... RNA was run in ... the electrophoresis ... n either and finally ... voltage of 100 V, ... cid and stained in 0.02 % toluidine blue for 60 min and de-stained in running cold tap water for 24—36 hrs. The migration was documented by densitometric tracing in a Joyce Loebl apparatus.

Determination of radioactivity in gel fractions

The gel was divided in two or three lengths (depending upon the number of migrations in the gel) a ... el razor blades ... ach sec ... t on b ... Each gel piece ... each gel section was put in liquid scintillation counting vials together with 10 ml of toluene containing 5 % Soluene (Packard), 0.5 % PPO and 0.05 % POPOP. In method 2, the vials were left at room temperature for 48 hrs and shaken twice during that period. In both cases the radioactivity was counted in a liquid scintillation spectrometer (Packard Tri Carb 3375). Quench correction was made for each sample and the radioactivity was then calculated as disintegrations per minute (DPM).

Results

The method of RNA extraction used proved to be suitable for ovarian tissue. RNA extraction of a tissue so rich in nuclei as the ovary naturally results in varying DNA contamination. With the use of composite agarose polyacrylamide electrophoresis, however, this contamination does not cause any greater disadvantage since the DNA band is stained red-blue with toluidine blue in contrast to the more purely blue RNA bands. In some cases DNase treatment was used as described above and a 30 min digestion of the extract resulted in complete disappearance of the DNA band. The DNase treatment also showed that the former DNA band did not mask any RNA fractions appearing as isolated bands in the investigations on this RNA type. The gel staining was informative also in regards to the amount of protein contamination since marked contamination resulted in blurring of the bands as well as in staining of irregular, often band-like strips parallel to the migration in the sections between the RNA bands. In some experiments ovaries were incubated with leucine-^3H or phenylalanine-^3H for 60 min, but no radioactivity was detected in the gels.



a

b

Fig 1 a Agarose polyacrylamide gel, stained with toluidine blue, photographed together with a metric scale The migration to the right represents liver RNA and the migration to the left represents ovarian RNA both run in the same gel for 60 min at 100 V, 35 mA

Fig 1 b Densitometric tracing (logarithmic scale) of the ovarian RNA fractions shown on the gel in Fig 1 a. The main RNA peaks are designated by their nominal numbers in Svedberg (S) units

The electrophoreses were run immediately after completion of the extraction procedure since signs of degradation in the RNA preparations were noticed already after two days in dissolved condition This degradation was not inhibited by pronase treatment and moreover, the extraction yielded less RNA when pronase was used

Ovarian RNA fractions

As seen from Fig 1, this method has a high resolution and offers a very good separation of the different RNA species Since the separation method is largely the same as previously described by Peacock and Dingman (1968) the different major RNA components in the present study refer to the same sedimentation numbers as used by these authors

The first detectable RNA fraction was heavier than 28S and more pronounced than the corresponding band of liver RNA The fraction corresponds to 8—10 % of the total ovarian RNA There exist two clear fractions of high molecular weight where the 28S band constitutes 50—55 % and the 18S constitutes 25—30 % of the total RNA In addition to these fractions there are at least two minor fractions between 28S and 18S The low molecular RNA is separated in 5S and 4S which amount to 3—4 % and 8—12 % respectively It appears that ovarian RNA has fewer fractions than liver RNA as shown in Fig 1

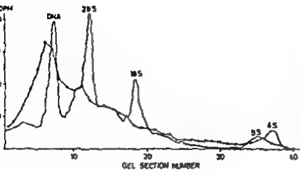


Fig. 2. Incorporation of radioactivity into the various RNA fractions after 30 min incubation. The ovaries were incubated in medium containing uridine-³H (0.1 mC/ml medium, specific activity 1760 mC/mmole). Electrophoretic conditions as in Fig. 1. Following densitometric tracing, the gel was cut into 40—60 sections. The radioactivity per gel section was determined, expressed as DPM and plotted in the figure together with the densitometric curve.

Labelling pattern of RNA from rat ovary

Twenty and thirty min *in vitro* labelling with uridine-³H gave a polydisperse picture (Fig. 2), with labelled fractions from higher than 28S down towards 4S. However, most of the radioactivity was bound to gel sections shortly before the DNA peak and thus to high molecular RNA fractions. A smaller activity peak was bound to the RNA band immediately prior to the 28S band, and there was also a small increase of activity in the 5S and 4S region.

Sixty min incubation also showed a polydisperse labelling pattern, (Fig. 3) most of the activity bound to RNA fractions of presumably somewhat lower molecular weight than in the 30 min labelling. Sixty min labelling resulted in comparatively more numerous activity peaks in the 28S—18S regions and the activity in the 5S region was more pronounced at this time, compared to 30 min incubation. After 240 min incubation of the ovaries most of the radioactivity was localized to the 4 major RNA fractions (Fig. 4). Addition of actinomycin-D to the incubation medium in a concentration of 100 μg/ml reduced the incorporation of uridine-³H with approximately 90 % (Fig. 5a and b). DNase treatment of RNA from shorter incubation periods did not change the distribution of activity maxima or the total counts.

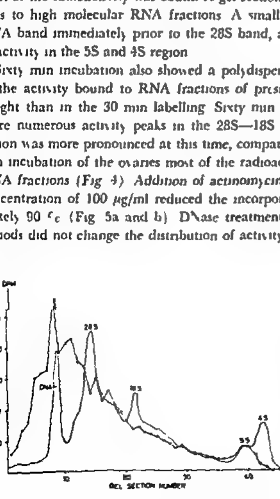


Fig. 3. Incorporation of radioactivity into the various RNA fractions after 60 min incubation. The ovaries were incubated in medium containing uridine-³H (0.1 mC/ml medium, specific activity 6700 mC/mmole). Electrophoretic conditions as in Fig. 1. Following densitometric tracing the gel was cut into 40—40 sections. The radioactivity per gel section was determined, expressed as DPM and plotted in the figure together with the densitometric curve.

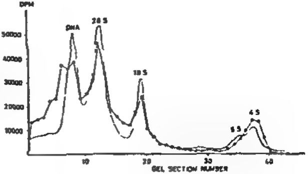


Fig 4 Incorporation of radioactivity into the various RNA fractions after 240 min incubation The ovaries were incubated in medium containing uridine ^3H (0 1 mC/ml medium, specific activity 6700 mC/mmole). Electrophoretic conditions as in Fig 1 Following densitometric tracing, the gel was cut into 40—60 sections The radioactivity per gel section was determined, expressed as DPM and plotted in the figure together with the densitometric curve

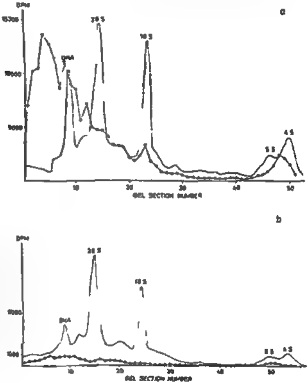


Fig 5 Effect of actinomycin D on the incorporation of radioactivity into the various RNA fractions The ovaries were first preincubated for 15 min in absence (a) or presence (b) of actinomycin D (100 μg/ml medium) The ovaries were then transferred to new flask containing uridine-^3H (0 1 mC/ml, specific activity 4250 mC/mmole), also now in absence (a) or presence (b) of actinomycin D, and incubated for 60 min Electrophoretic conditions as in Fig 1 Following densitometric tracing, the gel was cut into 40—60 sections The radioactivity per gel section was determined, expressed as DPM and plotted in the figure together with the densitometric curve

Comparisons between combustions according to Gupta (1966) and Soluene treatment revealed that the efficiency was of the same order in both cases while the recovery was slightly better with the use of Soluene scintillator

Discussion

The aim of the present project was to characterize with the utmost exactitude the ribonucleic acids in a tissue where little is known about the various RNA species present and where there is reason to expect a complex RNA pattern due to the

complexity of the tissue. It was therefore important to choose an extraction metho which gave a high yield of undegraded RNA in combination with a fractionatic method which gave high resolution and offers possibilities for rapid and reproducib analyses of large numbers of samples. Since the biological material available i studies on prepubertal rat ovaries is limited, a good recovery was another demand o the fractionation method.

The extraction method which was now found to be suitable for extraction o ovarian RNA is a modification of a method earlier used for extraction of brain RN (Egyhazi and Hydén 1966). For fractionation of RNA species, a modification of th electrophoretic method on agarose polyacrylamide gels described by Peacock an Dingman (1968) was used. Electrophoretic separation of RNA was first describe with agar as gel substance (Bachvaroff and McMaster 1964), and there has bee many indications that gel techniques are capable of higher resolution than the mor commonly used techniques of zone sedimentation and column chromatography. Ag gel electrophoresis seems to be most valuable in fractionation of high molecula weight RNA. A further development was achieved with the use of agarose instead o agar, when Tsanev (1965) described a method for separation of whole cell RN defining both high and low molecular RNA fractions. Electrophoretic separation o RNA on polyacrylamide gels was introduced by Richards and co-workers (Richard and Gratzer 1964; Richards, Coll and Gratzer 1965) who used gels with 5—10 % polyacrylamide in studies confined to fractionation of low molecular weight RNA. Ribosomal RNA, however, does not enter a 10 % polyacrylamide gel and barely a 5 % gel. Preparations of polyacrylamide gels of lower concentrations met with diffi culties since they did not retain sufficient strength to permit easy handling. Addition of small amounts of agarose to the polyacrylamide gels has solved these problems and composite agarose acrylamide gels were described by Uriel (1966) and Dingman and Peacock (1968). The most detailed description of RNA separation by electro phoresis on agarose-acrylamide gels has been given by Peacock and Dingman (1968). They showed that the mobility of the various RNA species on such composite gels was inversely related to the molecular weight of the RNA and they concluded that it was possible to obtain on a single electrophoretogram (1) qualitative information as to type and distribution of RNA species present, (2) quantitative data on isotope incorporation and (3) an estimate of the molecular weight of each species.

In the present study a composite gel with 2 % polyacrylamide and 0.5 % agarose was used and it was found that this type of gel gave a very good separation of both high and low molecular weight RNA of the prepubertal rat ovary. During the course of this work we also used the agarose method according to Tsanev (1965) but the resolution of the RNA fractions proved superior in the composite gel.

Upon electrophoresis the ovarian RNA separated in four well defined bands. Of these, the two slowest migrating bands can be assumed to represent the ribosomal RNA (Tsanev 1965; Peacock and Dingman 1967, 1968; Dingman and Peacock 1968) and are classified as 28S and 18S corresponding to the well known and well charac terized fractions of ribosomal RNA from rat liver migrating exactly the same dis

tance These two ovarian fractions were present in the expected ratio of [illegible] 1 or slightly higher

There were also at least two minor fractions between 28S and 18S Whether these fractions of probable ribosomal RNA represent intrinsically different kinds of RNA's or possibly artifacts of the methodological procedure, either during the incubation *in vitro* of the ovaries or during extraction and/or fractionation remains to be definitely settled in further studies It is of interest that Peacock and Dingman (1967) also reported new species of rat liver RNA with electrophoretic mobilities and sedimentation coefficients between 28S and 18S RNA and they presented also some evidence that these RNA components were present *in vivo* and were not the result of artifacts *in vitro* The above mentioned 28S/18S ratio in the ovarian RNA supports their evidence

Another characteristic pattern of the ovarian RNA was that there were RNA species of higher molecular weight than 28S and that these high molecular fractions were more pronounced than the corresponding liver RNA band

In the low molecular weight region of the ovarian RNA electrophoretogram two distinct bands were seen The electrophoretically faster moving RNA species corresponds to the 4S fraction of the liver RNA and this fraction represents 8—12 % of the total ovarian RNA The slower moving band representing 3—4 % of the total ovarian RNA corresponds to the 5S RNA described first by Rosset Monier and Julien (1964) for *Escherichia coli* and then for many types of mammalian cells (Richards *et al* 1965 Bachvaroff and Tongur 1966) It seems well-established from studies on bacteria and various mammalian cells that the 4S RNA fraction functions as transfer RNA but the function of the 5S RNA is still not clear Experimental data from mammalian cells (Bachvaroff and Tongur 1966) support the suggestion of Rosset Monier and Julien (1964) that the 5S RNA might be a structural component of the ribosomes

Twenty and 30 min labelling of ovarian RNA showed a polydisperse labelling ranging from fractions lower than 18S to much higher than 28S with maximum radioactivity concentrated to RNA fractions of molecular weight higher than 28S RNA This rapid labelling of a polydisperse high molecular RNA corresponds well to what has been found in liver RNA brain RNA and in nuclei from dipteran salivary glands (Vesco and Giuditta 1967 Jacob *et al* 1967 Edstrom and Daneholt 1967)

It is interesting to note that after 30 min incubation there is a relatively high labelling of the 5S—4S region higher than in gel sections immediately prior to 5S

After 60 min incubation there is still a polydisperse labelling with however increased radioactivity in the ribosomal fractions showing peaks associated to 28S and 18S and to fractions between those As in the previous experiments there is a definite labelling of 5S and 4S fractions The labelling of these low molecular RNA species was more pronounced after 60 min of incubation After 240 min incubation the labelling pattern had shifted to a more specific incorporation with radioactivity maxima bound to the major RNA peaks

The almost complete blockage of the RNA synthesis produced by actinomycin D indicates its DNA-dependence and is thus in line with numerous reports regarding the effect of this drug on other cell systems.

It must be kept in mind that these investigations were performed on whole ovaries and it is therefore difficult to refer the labelling to different cellular units, though it appears likely, in comparison with other studies, that the rapidly labelled fractions are derived mainly from nuclei (Edström and Daneholt 1967). The pattern with high labelling already after 30 min incubation with uridine-^{3}H also indicates that these fractions represent nuclear RNA.

It seems quite clear from the results of the present study that the extraction method used, as well as fractionation procedures by electrophoresis on agarose polyacrylamide gels are suitable methods for the further analysis and characterization of ovarian RNA. Studies are now in progress in our group to determine whether various gonadotrophic factors added acutely *in vivo* or *in vitro* to the prepubertal rat ovary influence the synthesis and/or the metabolism of different ovarian RNA species. In further studies, we also aim to investigate the RNA fractions in different cellular compartments in an effort to disclose the nature of rapidly labelled RNA in the ovary.

We are grateful to Dr B. Daneholt and Dr U. Ringborg, Karolinska Institutet, Stockholm, who introduced us to the gel electrophoretic techniques.

Valuable technical assistance was given by Miss Kristina Benetsson, Miss Barbro Berggren and Mrs Anita Sjogren.

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References

AHREN, K., L. HAMBERGER and LIDIA RUBINSTEIN: Acute *in vivo* and *in vitro* effects of gonadotrophins on the metabolism of the rat ovary. In *The Gonads*, Ed. McKerns, K. W. Appleton-Century-Crofts, New York 1969, pp. 327—354.

BACHVAROFF, R. and P. B. B. MCMASTER: Separation of microsomal RNA into five bands during agar electrophoresis. *Science* 1964, *143*, 1177—1179.

BACHVAROFF, R. J. and V. TONGUR: Ribonucleic acid in ribosomes from mammalian tissues. *Nature* (Lond.) 1966, *211*, 248—250.

GIVEN, M. C., B. BROWN and J. HILLIARD: Ribonucleic acid and protein synthesis in ovary. *Biochim. biophys. Acta* (Amst.) 1966, *144*, 127—134.

DINGMAN, C. W. and A. C. PEACOCK: Analytical studies on nuclear ribonucleic acid using polyacryl amide gel electrophoresis. *Biochemistry* 1968, *7*, 659—674.

DRURY, H. F.: Identification and estimation of pentoses in the presence of glucose. *Arch. Biochem.* 1948, *19*, 455—466.

VAN DYKE, K. and F. A. KATZMAN: Effect of pregnant mare's serum (PMS) on RNA polymerase activity and protein synthesis in ovaries of immature rats. *Endocrinology* 1968, *83*, 107—112.

EDSTRÖM, J. R. and B. DANEHOLT: Sedimentation properties of the newly synthesized RNA from isolated nuclear components of *Chironomus tentans* salivary gland cells. *J. molec. Biol.* 1967, *28*, 331—343.

EGYHAZI, E. and H. HYDEN: Biosynthesis of rapidly labeled RNA in brain cells. *Life Sciences* 1966, *5*, 1215—1223.

GUPTA, G. N.: A simple in-vial combustion method for assay of hydrogen-3, carbon-14 and sulfur-35 in biological, biochemical and organic materials. *Anal. Chem.* 1966, *38*, 1356—1359.

GUSTAFSSON, B E, Light weight stainless steel systems for rearing germfree rats *Ann N.Y Acad Sci* 1959 *78* 17—

JACOB, M, J SAMEC, J STEVENIN, J P GAREL and P MANDEL, Polysomes and polysomal RNA of rat brain *J Neurochem* 1967 *14* 169—178

JACOBY, F, Ovarian histochemistry. In *The Ovary* Ed Zuckerman, S Academic Press, New York, 1962 1 pp 189—

PE ... nucleic acid species by poly- ... 7

PE ... ion and separation of ribo- ... gels *Biochemistry* 1968 *7*.

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RICHARDS, E G and W B GRATZER, An investigation of soluble ribonucleic acid by zone electrophoresis *Nature* (Lond) 1964 *204* 878—879

RICHARDS, E G, J A COLL and W B GRATZER, Disc electrophoresis of ribonucleic acid in polyacrylamide gels *Analyt Biochem* 1965 *12*, 452—471

ROSSET, R. R MONIER and J JULIEN, Les ribosomes *d'escherichia coli Bull Soc Chim biol* (Paris) 1964 *46* 87—109

SAVARD, K, J M MARSH and B F RICE, Gonadotropins and ovarian steroidogenesis *Recent Progr Hormone Res* 1965 *21* 285—365

SCHNEIDER, W C, Determination of nucleic acids in tissues by pentose analysis In Methods in Enzymology Eds Colowick, S P and Kaplan, N O Academic Press Inc, New York, 1957 *III* 680—684

TSANEV, R, Direct spectrophotometric analysis of ribonucleic acid fractionation by agar gel electrophoresis *Biochim biophys Acta* (Amst) 1965 *103* 374—382

URIEL, J, Methode d electrophorèse sans des gels d acrylamide agarose *Bull Soc Chim biol* 1966 *48* 969—982

VESCO, C and A GUIDITTA, Pattern of RNA synthesis in rabbit brain *Biochim biophys Acta* (Amst) 1967 *142* 385—402

WIEME R J, In *Agar Gel Electrophoresis* Elsevier Publ Co, Amsterdam 1965 pp 70—71

Acta physiol. scand 1970. 79. 390—404

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Inhibition of Thromboplastin-Induced Intravascular Coagulation by Heparin and Trasylol

By

SAM NORDSTROM

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Abstract

NORDSTROM, S *Inhibition of thromboplastin induced intravascular coagulation by heparin and Trasylol* Acta physiol scand 1970 *79* 390—404

Homologous brain thromboplastin was injected or infused in different ways in dogs and various coagulation factors were followed for 7 days Slow infusions of thromboplastin in anesthetized dogs caused changes in platelet count and fibrinogen concentration similar to those usually seen in the postoperative period in man The heparin co-factor decreased only slightly which is contrary to the postoperative reactions in both man and dogs

The decrease in platelet count and fibrinogen concentration and the rate of thromboembol fter intravenous thromboplastin administration could partly be inhibited if the dogs were ated prophylactically with Trasylol and completely inhibited if they were pretreated with parin Trasylol also seemed to exert some slight influence on the antithrombin level in the rculating blood The in vivo anticoagulant effect of Trasylol might be the result of partial inhibition of more than one reaction in the coagulation process Compared to heparin Trasylol is a weak anticoagulant

The postoperative period involves increased risks for thrombotic complications In man major operations are followed by an immediate decrease and on the 1st—2nd day by an increase in platelet count fibrinogen concentration and factors V and VIII (Maslowski 1960 Egeberg 1962 Godal 1962 Blomback *et al* 1963 Amundsen *et al* 1963 Blomback Noren and Senning 1964) There is also a marked decrease in the plasma level of the heparin co-factor with a minimum on the 3rd to 5th postoperative days (Olsson 1963) These changes may be an indication of consumption coagulopathy Tissue thromboplastin released from injured tissues may be the triggering substance and consequently a possible cause of accelerated intravascular coagulation with postoperative thromboembolic and/or haemorrhagic complications (Penick *et al* 1958) Accordingly several investigations have been made on the effect of intravenously infused thromboplastin and thrombin in experimental animals (Ratnoff and Conley 1951 Hartmann Conley and Krevans 1951 Quick *et al* 1959 Lewis and Szeto 1962 Roberts *et al* 1964) However only the

immediate effect was studied and no attention was given to the changes in coagulation factors occurring after the first experimental day i e the time corresponding to the postoperative period

The postoperative decrease of the heparin co-factor in man can be completely or partly inhibited by pretreatment with heparin or the kallikrein inhibitor Trasylol (Olsson and Nordstrom 1964) In *in vitro* investigations, Trasylol has been shown to inhibit thromboplastin (Amris 1964, Blomback, Blomback and Olsson 1966) Mammen and Poucho (1966) and Nordstrom and Zetterqvist (1969) found a lower incidence of thromboembolism in Trasylol treated dogs than in dogs injected with thromboplastin alone These findings call for a more extensive investigation of the *in vivo* effect of Trasylol on thromboplastin-induced intravascular coagulation

The present paper deals with an investigation on changes in various coagulation factors during one week after different administration of homologous brain thromboplastin in dogs and a comparative study on the effect of pretreatment with heparin and Trasylol

Materials

Dog brain thromboplastin was prepared essentially according to Owren (1949) 6 thromboplastin preparations with concentrations of 5—33 arbitrary units (arb units)/ml were used in the experiments The calculation of the activity of each preparation was based on the con centration yielding a one stage prothrombin time of 18 5 sec This concentration was defined
Nordstrom 1969)
)I g/ml) were the commercial products

nine lung containing 5000 or 20 000

Methods

Hematocrit was determined in heparinized capillary tubes after centrifugation (Cellocrit AB L Ljungberg Stockholm)

Platelet count was determ ned essentially according to Kristenson (1922)

Fibrinogen concentration was measured according to Blomback and Blomback (1956) as described by Bergstrom Blomback and Kleen (1960) The error of the method (S D was 2 4 per en The

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Experimental procedure

Fifty-seven mongrel dogs weighing 8—23 kg were used for the experiments Nine of the dogs given infus ons were anesthetized by i v injection of thiopenthal sod um in doses of approximately 25 mg/kg b w the other 48 dogs were not anesthetized Thromboplastin was admin

[1] Internat onal units
[2] Kallikrein inhibitory units

TABLE I Experimental groups Range (mean value) of doses of thromboplastin (Thpl) Trasylol

Group		Number of dogs	Anesth	Thromboplastin inj time sec, min	dose rate arb u/kg/sec
I Thpl	a) short time i v inj	10		3—18	0 36—3 4 (1 6)
	b) long time i v inj	3		2—12	0 05—0 46
	c) i v infusion	2	+	70 90	0 001, 0 003
	d) s c inj	4			
II Tras + Thpl	a) short time i v inj (low Tras/Thpl)	8		4—14	0 71—3 6 (1 9)
	b) short time i v inj (high Tras/Thpl)	5		4—13	0 35—0 77 (0 46)
	c) i v infusion	2	+	80 90	0 001, 0 002
III Hep + Thpl	short time i v inj	5		5—11	0 21—0 57 (0 39)
IV Controls	a) short time i v inj of heated Thpl	3		10—12	2 4—3 8 (2 9)
	b) i v inj of Tras	4			
		2			
	c) i v inf of Tras	2	+		
	d) i v inj of Hep	4			
	e) i v inf of saline	3	+		

istered in different ways—by short time or long time intravenous injection (i v inj) of undiluted thromboplastin by infusion (inf) of thromboplastin in saline solution or by subcutaneous injection (s c inj) of undiluted thromboplastin Some dogs were treated i v with various doses of Trasylol or heparin prior to or in event of infusion simultaneously with thromboplastin The i v injections were given by puncture of a peripheral vein Dogs injected or infused with heated thromboplastin Trasylol heparin and saline respectively were used as controls

Blood samples were collected by venipuncture before anesthesia or thromboplastin administration and then on days 1 3 5 and in most of the dogs also on days 7 and 9 The blood was collected in siliconized glass tubes containing 3 8 % trisodium citrate (9 parts blood to 1 part citrate) for determination of fibrinogen concentration and heparin co-factor activity For platelet counting the blood was collected in tubes containing 5 mg of EDTA (disodium salt ethylendiamino-tetraacetic acid and for hematocrit determination in heparinized capillary tubes

Dogs surviving the experiment were killed 7 or 9 days later Autopsy was performed in all but two dogs At autopsy the caval veins the heart and the pulmonary arterial tree including subsegmental branches were thoroughly examined for thromboemboli

The dogs were divided in groups as shown in Table I Thromboplastin and Trasylol were [illegible] (Groups Ic IIc IVc) The infusion time of [illegible] IVe saline was infused for 90 180 min [illegible] time of Trasylol was between 3 and 10 min In [illegible] nd 30 min before and in Group IIb between 25 [illegible] In these groups the concentrations of Trasyl [illegible]

(Tras) heparin (Hep) and saline

total dose arb.u./kg	Trasylol KIU×10⁻³ per arb u Thpl	kg	Heparin IU×10⁻³ per arb u Thpl	kg	Saline ml/kg
2 5—31 7 (16 2)					
5 6—24 8					
4 0 14 3					15 30
1 6—27 6					
9 1—28 3 (16 1)	0 3—1 0 (0 55)	4—9 (7)			
1 7—10 0 (3 5)	2 0—4 0 (3 0)	4—35 (11)			
3 8 11 5	5 5 1 5	20 15			20 30
1 4—6 3 (3 3)			1—4 (2 8)	5 4—6 3 (5 9)	
24 0—45 8 (31 6)					
		3 5—39 (15)			
		100 100			
		11 33			30 30
				25—25	20—30
					25—30

at the moment of thromboplastin injection were calculated assuming that in dogs the initial half life of the inhibitory effect of i v injected Trasylol is about 30 min as in humans (Anderson Nilsson and Hedner 1967) In Groups IIc IVb and IVc the numerals refer to the injected or infused doses of Trasylol

In Group III heparin was injected 5 min before thromboplastin In three of the dogs the heparin was neutralized by i v administration of protamine chloride one hour after the heparin injection

In Group IVa the thromboplastin was heated in a water bath at 70° C for 15 min and cooled to room temperature before injection

Results

Mortality rate

None of the dogs injected with thromboplastin at an injection rate of less than 0 36 arb units/kg b w and sec died No relation was observed between the mortality rate and the thromboplastin dose or the changes in platelet count and fibrinogen concentration All dogs pretreated with heparin or with Trasylol at a dose ratio of more than 2000 KIU/arb unit thromboplastin survived

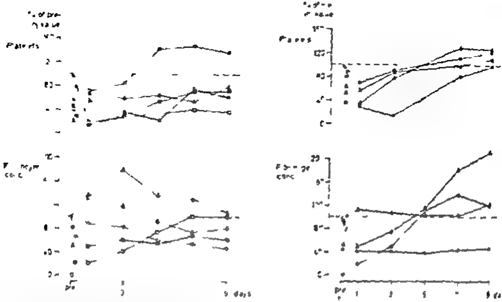

Fig 1 Intravenous injection or infusion of thromboplastin at different dose rates Effect on platelet count and fibrinogen concentration

△—△ dog 15 (anesth , Thpl dose 0 001 arb u /kg b w × sec — 4 0 arb ■ /kg b w
○—○ 11 0 05 — 5 6
□—□ 3 0 83 — 5 0
■—■ 4 0 83 —15 0
▲—▲ 16 (anesth 0 003 —14 3

ıg 2. Intravenous slow rate injection of very large amounts of thromboplastin and high rat… on of small amounts Effect on platelet count and fibrinogen concentration
△ dog 12 Thpl dose 0 27 arb u kg b w × sec —195 arb u /kg b w
▲ 13 0 46 —248
8 2 4 — 32
●—● 10 3 4 — 10

Thromboplastin (Group Ia) Four of the 10 dogs injected with thromboplastin during less than 20 sec died Two died within 4 hrs one during the first night and one 4 days later In these 4 dogs the dose injection rates of thromboplastin were 0 36 0 58 2 4 and 2 8 arb units kg b w and sec respectively and the total doses varied between 2 ɔ and 28 3 arb units kg b w

In the 6 surviving dogs the total thromboplastin dose ranged from 5 0 to 31 7 (mean 16 2) arb units kg b w and the injection rate from 0 83 to 3 4 (mean 1 7) arb units/kg b w and sec

Trasylol + thromboplastin (Group IIa) Three of the 8 dogs treated with Trasylol and thromboplastin at a dose ratio of less than 1000 KIU per arb unit died during the first night These three dogs had received large amounts of thromboplastin at a high injection rate (>2 4 arb units kg b w and sec) The total amount of thromboplastin varied between 10 0 and 28 3 arb units/kg b w

In the 5 surviving dogs the total thromboplastin dose ranged from 9 1 to 24 8 (mean 14 5) arb units/kg b w and the injection rate from 0 71 to 2 7 (mean 1 4)

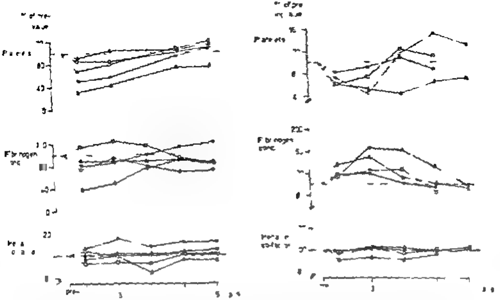

Fig 3 Intravenous injection (short time) of thromboplastin with and without pretreatment with Trasylol or heparin in surviving dogs and as control injection of heated thromboplastin Effect on platelet count fibrinogen concentration and heparin co-factor activity

Group IVa consists of 3 dogs, the other groups of 5 each The dose ranges are given in Tables I—IV The values of dog III in the thromboplastin group are not included in this graph

△—△	Group Ia	— Thpl alone	
○—○	IIa	— Tras + Thpl (550 KIU arb u)	
●—●	IIb	— Tras + Thpl (3 000 KIU arb u)	
□—□	III	— Hep + Thpl (300 IU arb u)	
▲—▲	IVa	— heated Thpl	
	Group Ia	— Thpl dose 1 4 arb u kg b w	sec —1 arb u kg b w
	IIa —	1 4	—15
	IIb —	0 46	— 3 5
	III	0 39	— 3 3
	IVa —	(2 9	—3?

Fig 4 Anesthetized dogs treated with intravenous infusion of saline Trasylol or of thromboplastin alone or simultaneously with infusion of Trasylol Effect on platelet count fibrinogen concentration and heparin co-factor activity

The saline group consists of 3 animals the other groups of 2 each The dose ranges are given in Table I

△—△ Group Ic

○—○ IIc

●—● IVc

□—□ IVe

arb units/kg b w and sec The mean dose ratio between Trasylol and thromboplastin remained at 550 KIU/arb unit.

Effect on platelet count

In Table II the values are given for surviving dogs after short time injection of thromboplastin alone or after pretreatment with Trasylol

Thromboplastin The decrease in platelet count seemed to be correlated rather to

TABLE II *Platelet count (10 ³/mm³) in surviving dogs given short time i v injections of thromboplastin alone or after pretreatment with Trasylol*

	Dog no	Trasylol KIU/ arb u	KIU/ kg	Thromboplastin arb u / kg × sec	arb u / kg	Pre inj value	Time after thromboplastin injection hrs 2	4	6	days 1	3	5	7	9
	3			0 83	5 0	300	116			58	85	—	124	110
	4			0 83	15 0	284	129		77	46	[illegible]	160	202	207
Ia	5			0 97	10 7	291	74		37	31	70	106	130	119
	6			1 8	24 8	187		163		160	252	—	255	248
	[illegible]			2 4	31 7	260		94		73	37	—	213	256
	10			3 4	10 2	348		276		242	316	—	393	433
	Mean			1 7	16 2	278				118	142	—	220	229
	30	500	4 000	0 71	10 0	377		238		199	224	370	313	451
	31	1,000	9 000	0 76	9 1	336		170		165	171	253	337	399
IIa	32	500	5 000	0 77	10 0	436		262		224	215	345	357	408
	33	300	8 000	2 1	24 8	290	278			295	310	—	333	375
	36	500	9 000	2 7	18 8	250		210		227	339	—	398	417
	Mean	*550*	*7,000*	*1 5*	*14 5*	*338*				222	252	—	348	399
	40	4 000	8 000	0 35	2 1	333	184			140	141	—	363	399
	41	2 000	4 000	0 37	1 9	312		175		100	99	—	260	375
IIb	42	2 000	4 000	0 38	1 9	312	239			260	300	—	340	377
	43	4 000	7 000	0 43	1 7	428	294			277	339	—	412	405
	44	3 000	33 000	0 77	10 0	221	84	106	88	74	98	174	167	234
	Mean	3 000	11 000	0 46	3 5	321				170	195	—	308	349

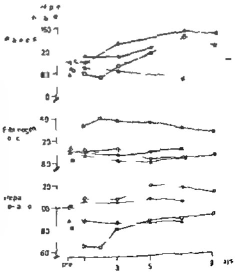

Fig 5 Intravenous injection of Trasylol and heparin and subcutaneous injection of thromboplastin *Effect on platelet count fibrinogen concentration* and heparin co-factor activity

The curve representing dogs treated with 100 000 KIU Trasylol per kg b w is formed by the mean values of [illegible] dogs the other curves by the mean values of 4 dogs each The dose ranges are given in Table I

△—△ Group Id — s c inj of Thpl dose 10 6 arb u /kg b w

○—○ IVb — Tras 15 000 KIU/kg b w

●—● IVb — Tras 100 000 KIU/kg b w

□—□ IVd — Hep 2 500 IU/kg b w

TABLE III Fibrinogen concentration (g/100 ml) in surviving dogs given short time i v injections of thromboplastin alone or after pretreatment with Trasylol

	Dog no	Trasylol KIU/ arb u	KIU/kg	Thromboplastin arb u / kg × sec	arb u / kg	Pre inj value	Time after thromboplastin injection hrs 2	4	[illegible]	days 1	3	5	7	9
	3			0 83	5 0	0 27	0			0 06	0 11	0 20	0 27	0 27
	4			0 83	15 0	0 38			0 08	0 19	0 23	0 21	0 26	0 23
Ia	5			0 97	10 7	0 42			[illegible]	0 14	0 13	0 30	0 29	0 30
	6			1 8	24 8	0 43		0 38		0 32	0 32	0 33	0 33	0 29
	[illegible]			2 4	31 7	0 26		[illegible]		0 05	0 13	0 29	0 36	0 31
	10			3 4	10 2	0 64		0 28		0 26	0 26	0 24	0 28	0 29
	Mean			1 7	16 2	0 40				0 17	0 20	0 26	0 30	0 28
	30	500	4 000	0 71	10 0	0 52		0 27		0 44	0 67	0 43	0 37	0 38
	31	1 000	9 000	0 76	9 1	0 49		0 20		0 32	0 36	0 33	0 28	0 34
IIa	32	500	5 000	0 77	10 0	0 30		0 15		0 22	0.28	0 24	0 27	0 27
	33	300	8 000	2 1	24 8	0 48	0 21			0 36	0 49	0 43	0 31	0 29
	36	500	9 000	2 7	18 8	0 32		0 27		0 26	0 27	0 26	0 22	0 22
	Mean	550	7 000	1 4	14 5	0 42				0 32	0 41	0 34	0 29	0 30
	40	4 000	8 000	0 35	2 1	0 35	0 16			0 25	0 33	0 30	0 29	0 26
	41	2 000	4 000	0 37	1 9	0 15		0 04		0 17	0 17	0 18	0 19	0 20
IIb	42	2 000	4 000	0 38	1 9	0 28	0 18			0.26	0 30	0 32	0 36	0 34
	43	4 000	7 000	0 43	1 7	0 28	0 09			0 17	0 18	0 24	0 37	0 53
	44	3 000	33 000	0 77	10 0	0 33			0 10	0.24	0 22	0 31	0 33	0 28
	Mean	3 000	11 000	0 46	3 5	0 28				0 22	0 24	0 27	0 31	0 32

the injection rate of thromboplastin than to the total amount of i v injected thromboplastin (Fig 1—2) When given slowly even very large total thromboplastin doses influenced the platelet count only moderately (Fig 2) The moderate platelet decrease in dog 10 may be due to the short injection time of only 3 sec, preventing a sufficiently high concentration of thromboplastin being maintained for sufficiently long time

Subcutaneous administration of thromboplastin and i v injection of heated thromboplastin did not cause any significant changes in platelet count (Fig 3 and 5)

Trasylol + thromboplastin In dogs treated with comparable doses of thromboplastin (Groups Ia and IIa) the decrease in platelet count seemed to be smaller with Trasylol pretreatment than without (Fig 3 Table II) This could, however not be statistically verified (day 1 $0\,05 < p < 0\,1$ day 3 and 7 $0\,1 < p < 0\,2$) In order to obtain comparable thromboplastin dose rates dog 10 was excluded from the thromboplastin group (Ia) shown in Fig 3 Raising the quotient Trasylol/thromboplastin from 550 to 3000 KIU Trasylol/arb unit of thromboplastin did not cause a stronger inhibition of the platelet reaction On the contrary, the fall in platelet count was more pronounced in dogs with the higher Trasylol/thromboplastin ratio (Group IIb)

TABLE IV Heparin co factor activity (per cent of activity in standard plasma) in surviving dogs given short time i v injections of thromboplastin alone or after pretreatment with Trasylol

	Dog no	Trasylol KIU/ arb u	KIU/kg	Thrombo-plastin arb u / kg × sec	arb u / kg	Pre-inj value	Time after thromboplastin injection hrs 2	4	6	days 1	3	5	7	9
	3			0 83	5 0	116	92			112	120	85	112	106
	4			0 83	15 0	106			110	80	102	88	110	96
Ia	5			0 97	10 7	76			88	84	111	62	70	76
	6			1 8	24 8	124		116		116	100	126	124	126
	8			2 4	31 7	116		124		132	128	100	106	110
	10			3 4	10 2	98		90		90	90	98	104	104
	Mean			1 7	16 2	106				102	104	93	104	103
	30	500	4 000	0 71	10 0	102		112		94	112	102	98	102
	31	1 000	9,000	0 76	9 1	88		104		88	88	98	94	94
IIa	32	500	5 000	0 77	10 0	86		92		94	92	82	111	88
	33	300	8 000	2 1	21 8	116	124			128	92	108	116	116
	36	500	9 000	2 7	18 8	120		120		130	124	120	138	138
	Mean	550	7 000	1 4	14 5	102				107	102	102	106	108
	40	4,000	8,000	0 35	2 1	112	134			130	138	138	142	130
	41	2,000	4,000	0 37	1 9	84		98		84	106	111	84	106
IIb	42	2 000	4 000	0 38	1 9	110	116			120	124	112	116	102
	43	4,000	7,000	0 43	1 7	86	98			112	106	111	110	106
	44	3 000	33 000	0 77	10 0	120			122	98	110	114	98	120
	Mean	3,000	11,000	0 46	3 5	102				109	117	110	110	113

In anesthetized dogs infused with thromboplastin of low concentration, the platelets seemed to return earlier to the initial level if the dogs were infused with Trasylol simultaneously with the thromboplastin-saline infusion (Fig 4)

After i v injections of Trasylol alone a slow increase in platelet count was obtained with doses of 3000—100,000 KIU/kg b w (Fig 5)

Heparin + thromboplastin The decrease in platelet count was almost completely inhibited if the dogs were heparinized prior to the thromboplastin injection (Fig 3) No difference occurred between dogs treated with or without protamine chloride

Heparin alone injected in a dose of 2500 IU/kg b w caused the same slight increase in platelet count as after injection of Trasylol alone (Fig 5)

Effect on fibrinogen concentration

Thromboplastin There seemed to be a correlation between the amount of thromboplastin injected per sec and the decrease in fibrinogen concentration during the first days (Fig 1—2, Table III) Slow rate injections of thromboplastin (below 0 36 arb unit/kg b w and sec) caused slight or no decrease in fibrinogen concentration After

large thromboplastin doses, supernormal fibrinogen values were obtained from the 5th day onwards

After thromboplastin infusions in anesthetized dogs, the fibrinogen concentration had increased already the first day and reached a higher level than in dogs infused with saline only (Fig 4)

Heated thromboplastin and subcutaneously injected thromboplastin did not cause any significant changes in fibrinogen concentration (Fig 3 and 5)

Trasylol + thromboplastin Small and large doses of Trasylol seemed to inhibit the fall in fibrinogen concentration to about the same degree (Fig 3, Table III) In dogs treated with comparable doses of thromboplastin (Groups Ia and IIa), the difference is statistically significant ($0.001 < p < 0.01$) days 1—3 between dogs given Trasylol and non pretreated dogs

I v injections of Trasylol alone in doses up to 100 000 KIU/kg b w had no influence on the fibrinogen concentration (Fig 5)

Heparin + thromboplastin Pretreatment with heparin prevented the decrease in fibrinogen concentration after i v thromboplastin injection (Fig 3) Subsequent injection of protamine chloride did not alter the result Injection of heparin alone caused an increase of the fibrinogen concentration in all four dogs (Fig 5)

Effect on fibrinolytic activity

Thromboplastin In dog 12, given a very large total thromboplastin dose, increased fibrinolytic activity (up to 247 μg/ml/hr) was recorded in the circulating blood between the 3rd and 7th day In two anesthetized dogs thromboplastin infusion during 70—90 min was followed by moderately increased activity on [illegible] out of 10 occasions during the first [illegible] days (Group Ic) In the other dogs only single values exceeded the normal limit

After infusion of saline alone in three anesthetized dogs increased fibrinolytic activity was recorded on 4 out of 11 occasions during the first 7 days (Group IVe)

Trasylol + thromboplastin Even when Trasylol was infused simultaneously with thromboplastin during 70—90 min in two anesthetized dogs (Group IIc), the same frequency of increased fibrinolytic activity occurred during the following 9 days (7 out of 10 occasions) In two anesthetized dogs infused with Trasylol alone (Group IVc) hyperfibrinolysis also appeared (3 out of [illegible] occasions) Otherwise no hyperfibrinolysis was observed in dogs given Trasylol alone or together with thromboplastin

Heparin + thromboplastin Heparinization with or without thromboplastin injection did not seem to influence the fibrinolytic activity during the subsequent week

Effect on heparin co factor activity

Thromboplastin No correlation to the thromboplastin dose was obtained In most dogs there were slightly decreased values the minimum occurring irregularly between the 1st and 7th day (Table IV) The mean curve for 5 of the dogs is shown in Fig 3

In anesthetized dogs, even small amounts of thromboplastin caused some slight decrease in heparin co factor activity (Fig 4) Infusion of saline had no effect on the activity

Injection of heated thromboplastin did not influence the activity of the heparin co factor (Fig 3) Subcutaneously injected thromboplastin decreased the co factor activity by 10—15 per cent during days 1—7 (Fig 5)

Trasylol + thromboplastin In practically all dogs given Trasylol with or without subsequent thromboplastin injection the heparin co-factor activity remained at or above the pre injection value for at least 1 week (Table IV, Fig 3 4 5) With comparable thromboplastin doses the co factor values of dogs pretreated with Trasylol (Group IIa) were almost significantly higher on day 5 ($0.01<p<0.05$) than in dogs given thromboplastin alone (Group Ia)

Heparin + thromboplastin In heparinized dogs thromboplastin caused a slight decrease in co factor activity of about the same degree as in dogs injected with thromboplastin alone (Fig 3) Heparin alone however decreased the co-factor activity by 35 per cent already on the first day (Fig 5)

The heparin co factor activity did not seem to be correlated to changes in platelet count and fibrinogen concentration

Effect on hematocrit

In anesthetized dogs infused with saline thromboplastin or thromboplastin together with Trasylol there was an increase in hematocrit reaching its maximum on the 5th

This was about 20 per cent above the pre infusion value In dogs injected with thromboplastin after pretreatment with heparin no changes in hematocrit was observed In all the other non anesthetized dogs the hematocrit decreased by 5—10 per cent with the lowest value on days 3—5

Rate of thromboemboli

Autopsies were performed in all but 2 dogs The latter were given thromboplastin subcutaneously Macroscopical thromboemboli were found in the right side of the heart and the pulmonary arteries only in Groups Ia IIa and IIb

Thromboplastin (Group Ia In 5 of the 6 dogs which survived the experiment and were killed 9 days later small to massive thromboemboli had occurred All four dogs who died during the experiment showed thromboemboli three of which occluded the outflow of the right ventricle or the pulmonary artery

Trasylol + thromboplastin (Group IIa) No thromboemboli were found in the five surviving dogs Two of the three dogs which died showed occluding thromboemboli whereas in one no emboli were observed

Thus in the groups with comparable thromboplastin doses thromboemboli occurred in 9 out of 10 untreated dogs and in 2 out of 8 Trasylol treated dogs

Of the five surviving dogs treated with the high Trasylol thromboplastin ratio (*Group IIb*), two had small mural clots on the tricuspidal valves whereas in the other three none could be detected

Discussion

As in previous animal studies, the decrease in platelet count and fibrinogen concentration was more pronounced and prolonged after rapid than after slow injections of thromboplastin (Ratnoff and Conley 1951, Hartmann, Conley and Krevans 1951 Adelson Rheingold and Parker 1960, Lewis and Szeto 1962, Roberts *et al* 1964 Rapaport *et al* 1966) Relatively small total amounts of thromboplastin if given rapidly, could be sufficient for consumption of platelets and fibrinogen and thrombus formation, while slow injections of large amounts of thromboplastin seemed to be less harmfull This difference might be due to the reticuloendothelial system (RES) having a limiting capacity for clearance of thromboplastin and/or fibrin On the other hand, high doses given during very short times might be relatively harmless due to rapid clearance of the thromboplastin activity in the reticuloendothelial system (compare dog 10) The critical thromboplastin concentration however, may vary in the individual dogs due to differences in blood flow rate anticoagulant activity of plasma fibrinolytic activity, RES blockade and general condition These differences could explain the poor correlation between thromboplastin dose on one side and the rate of thromboemboli and mortality on the other

During the first week those anesthetized dogs which were infused with thromboplastin during 1—1 1/2 hrs, developed hyperfibrinogenemia already from the 1st day This is in contrast to the fibrinogen reaction after thromboplastin injection at high dose injection rate The slow thromboplastin infusions may simulate conditions during operations after which supernormal fibrinogen values are a regular finding (Godal 1962) The nature of this postoperative increase of plasma fibrinogen is unclear One explanation could be that thromboplastin released during operation or slowly infused will cause reactions leading to a stimulation of fibrinogen synthesis During short term experiments it has been shown that *de novo* fibrinogen synthesis occurs within a few hours after administration of thrombin and thromboplastin (Nordstrom and Zetterqvist 1968 1969) The same mechanism might operate in these long term experiments Thromboplastin in high concentrations might also enhance catabolic processes or exert a toxic effect on fibrinogen synthetising cells and on the platelet forming system This would explain the late re appearance of fibrinogen and platelets after rapid injections of large doses of thromboplastin

Irrespective of the mode of thromboplastin administration the heparin co-factor decreased only by 10—20 per cent This is different from the postoperative course both in man (Olsson 1963) and in dogs (Nordström, unpublished data) where a marked decrease of 50—70 per cent is a regular observation Olsson suggested that the antithrombin was utilized in a process by which thrombin or thromboplastin activity were inactivated However in the present investigation, no correlation could be recorded between the heparin co factor and the thromboplastin dose This discrepancy might indicate that the factor(s) responsible for the postoperative co-factor decrease are not present in the thromboplastin preparations used in this study

In heparinized dogs thromboplastin produced no thromboembolic phenomena or

any marked decrease in platelet count and fibrinogen concentration Trasylol compared to heparin is *in vitro* a weak antithromboplastin (Amris 1964 Blomback Blomback and Olsson 1967, Nordstrom 1969) In dogs injected with thromboplastin however it was possible by pretreatment with Trasylol to decrease markedly the rate of thromboembolic complications This thrombus protective effect of Trasylol was also found by Mammen and Poucho (1966) The less marked decrease and earlier return of platelets and fibrinogen in the Trasylol treated animals as found in this study also suggests that Trasylol has an *in vivo* anticoagulant effect This *in vivo* effect of Trasylol is somewhat astonishing in view of its relatively weak effect *in vitro* However *in vivo* Trasylol might act differently than *in vitro* The possibility of Trasylol as a broad proteinase inhibitor interfering with different reactions in the coagulation process must be taken into account too According to Mammen and Poucho (1966) the action of Trasylol is directed against the final phase of coagulation causing an impaired fibrin clot formation The result of the present investigation does not contradict such an effect of Trasylol

The complex action of Trasylol is further demonstrated by the findings of Dubber *et al* (1968) They reported that accelerated platelet aggregation occurs 30 min after a single intravenous injection of Trasylol a finding in apparent discord with the *in vitro* observations that Trasylol inhibits platelet aggregation Correlated to the Trasylol dose the speed of platelet aggregation increased with defective thromboplastin generation A higher speed of platelet aggregation at the time of thromboplastin injection is a possible explanation of the findings in the present investigation a more marked fall in platelet count in dogs pretreated with large doses of Trasylol than with small

Godal and Skaga (1967) reported that moderate doses of Trasylol counteract the rise in plasma fibrinogen following major surgery They suggested that Trasylol had an influence on the metabolism of fibrinogen In the present investigation the rise in fibrinogen that was observed in anesthetized dogs infused with thromboplastin could not be prevented by pretreatment with Trasylol The influence of Trasylol on the metabolic pathways of fibrinogen after thromboplastin administration might be different from those in the postoperative period In this connection it is noteworthy that increased fibrinogen values appeared after heparin administration alone As this phenomenon did last for several days it can hardly be explained by an inhibition of a permanent coagulation process occurring in the circulating blood Another explanation could be that heparin interferes with the synthesis or catabolism of fibrinogen

The marked fall of the heparin co factor after injection of heparin alone was described by Olsson (1963) and explained as being caused either by a firm complex formation between heparin and its co factor or by heparin catalyzing a reaction by which an active antithrombin (co factor) is formed from a precursor The complex or the active antithrombin might rapidly be eliminated from the blood When thromboplastin was injected after heparin the co factor decreased however only to a small degree indicating a competition for heparin between the co-factor and thromboplastin

In the present experiments, Trasylol seemed to prevent the small heparin co-factor decrease following i v administration of thromboplastin Trasylol alone caused a small increase of the co-factor activity This is in conformity with the observation from the *in vivo* administration of Trasylol during surgical interventions in man (Olsson and Nordström 1964) The present *in vivo* experiments are in contrast to the effect of Trasylol *in vitro*, where no influence on the antithrombin system of plasma has been observed (Godal and Theodor 1965, Nordström 1969) This suggests that the effect of Trasylol on the antithrombin activity is indirect

Trasylol, which is shown to be a potent antifibrinolytic substance (Marx *et al* 1959 Godal and Theodor 1965), had no influence on the slightly increased fibrinolysis which appeared in anesthetized dogs after slow infusion of thromboplastin and of saline Anesthesia or fluid infusion or a combination of both might, therefore, give rise to a type of hyperfibrinolysis uninfluenced by Trasylol or appearing after the period during which Trasylol is effective

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References

ADELSON E J J RHEINGOLD and O PARKER Platelet and fibrinogen sequestration *Blood* 1960 *15* 596—605

AMRIS C J Inhibition of fibrinolytic and thromboplastic activity by Trasylol *Abstr Xth Congr Int Soc Haemat* 1964 G 72 (*Scand J Haemat* 1966 *3* 19—32)

AMUNDSEN M A J A SPITTELL JR H THOMPSON JR and C A OWEN JR., Hypercoagulability associated with malignant disease and with the postoperative state Evidence for

A

BERGSTROM K B BLOMBACK and G KLEEN Studies on the plasma fibrinolytic activity in a case of liver cirrhosis *Acta med scand* 1960 *168* 291—305

BLOMBACK, B and M BLOMBACK Purification of human and bovine fibrinogen *Arkiv Kemi* 1956 *10* 415—443

BLOMBACK B M BLOMBACK H LAGERGREN and P OLSSON The heparin co-factor activity in plasma and its relation to the anticoagulant effect of intravenously injected heparin *Acta physiol scand* 1963 *58* 306—318

BLOMBACK B M BLOMBACK and P OLSSON Action of a proteolytic enzyme inhibitor on ... ylol Therapie F K Schattauer Verlag ... emorrh (Stuttg) 1967 *18* 190—197

... TTERQVIST Effekt av operationstrauma och koagulationsfaktorer *Nord Med* 1963 *69* 155

BLOMBACK M I NOREN and A SENNING Coagulation disturbances during extracorporeal circulation and the postoperative period *Acta chir scand* 1964 *127* 433—445

DUBBER A H C G P MCNICOL E UTTLEY and A S DOUGLAS *In vitro* and *in vivo* studies with Trasylol an anticoagulant and a fibrinolytic inhibitor *Brit J Haemat* 1968 *14* 31—49

EGEBERG O Changes in the coagulation system following major surgical operations *Acta med scand* 1962 *171* 679—685

GODAL, H C, Quantitative and qualitative changes in fibrinogen following major surgical operations *Acta med scand* 1962, *171*, 687—691
GODAL, H C and [illegible] after major surge[illegible]
GODAL, H C anc[illegible] Trasylol and by [illegible] 199—208
HARTMANN, R C, C L CONLEY and J R KREVANS, The effect of intravenous infusion of thromboplastin on "heparin tolerance" *J clin Invest* 1951 *30* 948—956
KRISTENSON, A, A new method for the direct counting of the so-called blood platelets in man *Acta med scand* 1922 *57* 301—312
LEWIS, J H and [illegible] ment of an anti[illegible]
MAMMEN, E F a[illegible] blood in dogs [illegible] *Ann N Y Acad Sci* 1968 *146* 713—714
MARX, R, P CLEMENTE, E WERLE and W APPEL, Zum Problem eines Antidotes in der internen Thrombotherapie mit Fibrinolytika *Blut* 1959 *5* 367—375
MASLOWSKI, W C, The effect of surgery on blood coagulation *J oral Surg Anesth* 1960 *18* 97—107
NORDSTROM, S, Inhibition of blood coagulation by Trasylol *Coagulation* 1969 In press
NORDSTROM, S and E ZETTERQVIST, Effects of thrombin infusion upon ^{131}I labelled fibrinogen in dogs *Acta physiol scand* 1968 *72* 85—99
NORDSTROM, S and E ZETTERQVIST, Effects of epsilon aminocaproic acid (EACA) and Trasylol on thromboplastin induced intravascular coagulation, studied in dogs with iodine labelled fibrinogen *Acta physiol scand* 1969 *76* 93—105
OLSSON, P, Exogenous heparin and the heparin co-factor *Acta chir scand* 1963 *Suppl 319*
OLSSON, P and S NORDSTROM, The significance of postoperative variations in antithrombin *Thoraxchirurgie* 1964 *12* 176—180
OWREN, P, A quantitative one stage method for the assay of prothrombin *Scand J clin Lab Invest* 1949 *1* 81—83
PENICK, G D, H R ROBERTS W P WEBSTER and K M BRINKHOUS, Hemorrhagic states secondary to intravascular clotting *Arch Path* 1958 *66* 708—714
QUICK, A J, C V HUSSEY, J HARRIS and K PETERS, Occult intravascular clotting by means of intravenous injection of thrombin *Amer J Physiol* 1959 *197* 791—794
[illegible]PAPORT, S I, P F HJORT M J PATCH and M JEREMIC, Consumption of serum factors and prothrombin during intravascular clotting in rabbits *Scand J Haemat* 1966 *3* 59—75
RATNOFF O D and C L CONLEY, Studies on afibrinogenemia II The defibrinating effect on dog blood of intravenous injection of thromboplastic material *Bull Johns Hopk Hosp* 1951 *88* 414—424
ROBERTS, H R, G D PENICK, W P WEBSTER and K M BRINKHOUS, The prevention of hemorrhage with anticoagulants — an exploratory study of a paradox *Ann N Y Acad Sci* 1964 *115* 67—77

Acta physiol. scand. 1970. 79. 405—410

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Dopamine Secretion from the Isolated Perfused Sheep Adrenal

By

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Abstract

Lishajko F *Dopamine secretion from the isolated perfused sheep adrenal* Acta physiol scand 1970 *79* 405—410

Isolated sheep adrenals, perfused with Tyrode solution, release in addition to adrenaline (A) and noradrenaline (NA) small amounts of dopamine (DA) into the effluent The amines [illegible] Addition of ACh to the per- [illegible] DA about 5 times In calcium [illegible] 7—2 7 times for all 3 amines [illegible] to ACh and carbachol 5—7 times Injection of Ca^{2+} into the perfusion fluid further increased the secretory response to simultaneously added ACh and carbachol 14—18 times for all 3 amines

It is well-known that ACh as physiological neurotransmitter stimulates the chromaffin cells to secrete adrenaline (A) as well as noradrenaline (NA) Butterworth and Mann (1957) have reported that repeated i v injections of ACh cause depletion of catecholamines from the adrenal gland *in vivo* Douglas and Rubin (1961 1963) have shown on isolated perfused adrenals that the secretion of catecholamines by ACh requires the presence of Ca^{2+} ions in the media Philippu and Schumann (1962) have confirmed this finding

In addition to A and NA the adrenal medulla contains dopamine (DA) as discovered by Goodall (1951) in the sheep adrenal later confirmed by Shepherd and West (1953) for bovine adrenals and in the dog by Puppi *et al* (1965) Eade (1958) has shown that the DA in the bovine adrenal was bound to a 'large granules' fraction

Recently it has been shown (Lishajko 1968 1969) that isolated granules from sheep adrenal medulla contain DA and that this amine behaves differently from A and NA as regards release reuptake and net uptake Secretion of labelled H^3-DA as well as of H^3-NA *from the adrenal gland after i v injection of labelled H^3-1-tyrosine* in the cat has been reported by Hempel and Mannl (1967, 1969)

In this communication it will be shown that DA is secreted from the isol [illegible] r-

fused sheep adrenal concomitantly with NA and A which has not been reported previously Secretion occurs spontaneously to a small extent and is greatly enhanced by stimulation by ACh or carbachol and calcium ions

Methods

Sheep adrenal glands were obtained immediately after the sacrifice of the animals Four sheep adrenals were used for the experiments After dissection of the gland (weight about 2 g) incisions were made between 0 1—0 3 mm deep Retrograde perfusion was made through the vein using a constant flow pump (Sigmamotor) at a rate of 2 ml/min The Tyrode solution used had the following composition (mM) NaCl 137 KCl 2 7, $CaCl_2$ 1 8 $MgCl_2$ 0 11 $NaHCO_3$ 11 9 NaH_2PO_4 0 4 and glucose 5 6 In other experiments the chloride ions were replaced by acetate (acetate Tyrode) (mM) NaAc 137 KAc 2 7 $Ca(Ac)_2$ 1 8 $Mg(Ac)_2$ 0 11 $NaHCO_3$ 11 9 NaH_2PO_4 0 4 glucose 5 6 and HAc, 1 mM pH about 7 2 In Ca^{2+} free Tyrode $Ca(Ac)_2$ was replaced by equimolar NaAc The perfusion fluid was gassed with 95 % O_2 + 5 % CO_2 at 37° C Secretion of NA A and dopamine (DA) was stimulated by injection of 40—80 μg acetylcholine or carbachol in 1—5 ml perfusion fluid during 1—2 min through a polyethylene catheter close to the cannulated vein The effluent was collected continuously during 2 5 min periods in glass cylinders containing 0 3 ml 4 N perchloric acid

After centrifugation of the perfusate for 5—10 min at 10 000 × *g* the clear extract was used for analysis of NA and A fluorimetrically A number of fractions were run on Dowex 50 × 4 in Na form for separation of NA A and DA To 2 ml of extract was added 5 ml water + 6 mg EDTA and pH brought to 4 5 with 1 N KOH After removal of the perchlorate precipitate by centrifugation the supernatant was used for separation of NA A and DA according to Carlsson and Waldeck (1958) Good agreement was obtained on analysis of A and NA before and after separation on Dowex 50 × 4

Identification of DA in effluent

Aliquots of the eluate after separation on Dowex 50 × 4 were used for identification of DA on Aminco Bowman spectrophotofluorimeter connected with a recorder

Results

Normal Tyrode solution

Using normal Tyrode solution as perfusion medium the spontaneous secretion of A NA and DA gradually declines to an approximately constant level Fig 1 shows the decrease of secretion of A from 18 μg/min at the start to 5 μg/min after 12 min perfusion NA and DA secretion were reduced from 2 3 and 0 6 μg/min to 1 0 and 0 11 μg/min respectively after 12 min

After stimulation of the chromaffin cells by 50—80 μg ACh during 2 min periods the secretion of A and NA increases 1 5—2 5 times while DA increases about 5 times in comparison with the previous control fraction (5) before stimulation and amounted to 11 6 μg A 2 2 μg NA and 0 54 μg DA/min Secretion of all 3 amines decreases rapidly after stimulation The proportions of the secreted A NA and DA were about the same 80 16 and 4 per cent respectively before and after stimulation by ACh

Acetate Tyrode, without Ca^{2+}

The spontaneous secretion of A and NA from the gland perfused with acetate Tyrode with or without Ca^{2+} ions also decreases progressively during perfusion of the gland A slight decrease occurred in the secretion of NA in proportion to the total (NA + A + DA) during the first 20 min of perfusion (Fig 2)

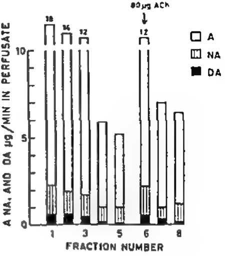

Fig 1 Sheep adrenal gland perfused with normal Tyrode 2 ml per min at 37° C 80 µg ACh in 5 ml Tyrode during 2 min at fraction (6)

Addition of 40 µg ACh and of 40 µg carbachol (fraction 14) increased the secretion of NA and A 1 7—2 7 times when Ca" -free acetate-Tyrode was used for perfusion (cf Fig 2) About 6 per cent of the total amines was DA, and 30 per cent NA in the perfusate which was approximately the same as before carbachol stimulation

Acetate-Tyrode with normal Ca^{2+} content

Reintroduction of 1 8 mM $Ca(Ac)_2$ to the acetate-Tyrode (fractions 55 and 60 Fig 2), increased the responses to ACh or carbachol for all 3 amines to about 5—7 times the amount during the prestimulation period (Table I)

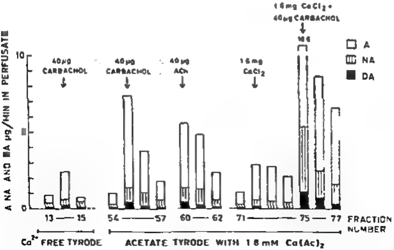

Fig 2 Sheep adrenal gland perfused with acetate Tyrode in pH 7 2 at 37° C without and with Ca^{2+} ions 40 µg ACh or 40 µg carbachol (fraction 14, 55, 60) at fraction 72 addi 1 6 mg $CaCl_2$, at fraction 75 1 6 mg $CaCl_2$ + 40 µg carbachol in 1 ml perfusion medium 1 min as indicated Effluent collected during 2 5 min periods for each fraction

TABLE I Amine secretion from perfused sheep adrenal gland ug/m n (mean and range) before and after stimulation by acetylchol ne

n = 3	DA	NA	A
Bef re Stimulation	0 14 (0 08—0 28)	0 44 (0 18—0 96)	1 4 (0 54—2 5)
After Stimulation	0 75 (0 43—1 4)	2 2 (1 4—3 9)	9 9 (7 4—16 7)

A slight decrease in the proportion of NA and an increase of A was noted during stimulation by ACh or carbachol followed by a return to the same level as before stimulation The proportion of DA did not change Omission of phosphate ions from the acetate Tyrode caused no difference in the secretion of NA and A during stimulation by 40 μg ACh

Effect of increased Ca^{++} and Ca^{++} together with carbachol

Injection of 1 6 mg $CaCl_2$ alone (fraction 72) in 2 ml acetate Tyrode increased the secretion of A NA and DA about 2 1 5 and 2 times that of the previous fraction Injection of 1 6 mg $CaCl_2$ together with 40 μg carbachol (fraction 75) in 2 ml acetate Tyrode during 2 min increased the secretion 18 18 and 13 7 times the pre stimulation value (fraction 75) (Fig 2) or considerably more than the sum of the increase observed for Ca^{++} or carbachol alone

Identification of DA in perfusate

In order to identify DA in the perfusate (fraction 76 Fig 2) authentic DA was used for comparison The activating and fluorescence spectra of the secreted DA in the perfusate are shown in Fig 3 in comparison with standard DA Fraction 14 (Fig 2) which was stimulated by carbachol in Ca^{++} free Tyrode also shows similar characteristics in agreement with authentic DA

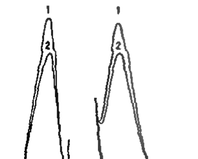

Fig 3 Fluorescence spectra of DA in effluent from fraction 76 (Fig 2) and of standard DA Aliquot of eluate after separation of DA on Dowex 50×4 corresponding to 0 42 μg DA (lower curve) and 0 5 μg standard DA (upper curve) (after oxidation and transformation to the fluorophores) Curves number DA 1 eluate 2 DA blank 3 and eluate blank 4
Activating 340 mμ and fluorescence 395 mμ (non corrected) wave length Aminco Bowman instrument

Discussion

The results in this study confirm the findings obtained by Douglas and Rubin (1961, 1963) Philippu and Schumann (1962), Stjarne (1964), Kirshner *et al* (1966) and others that NA and A secretion in isolated perfused adrenal gland is increased by ACh and Ca^{++} ions in the media The secretion of a small but regular amount of DA in addition to NA and A demonstrated in the present report, is also stimulated by ACh or carbachol and increased by Ca^{++}. This response is seen also when chloride ions were replaced by equimolar acetate

The fact that DA is found in chromaffin cell granules suggests that it is an independent cell hormone and not only a precursor to the other amines In addition it seems unlikely that the proportion secreted DA would be parallel to NA and A during rest as well as after stimulation if DA was mainly a precursor The varying and sometimes high amounts of DA found in different tissues (Euler and Lishajko 1957, Bertler *et al* 1959 1960) also speak in favour of an autonomous function DA is present either in specific cells (Falck *et al* 1959 a, b) or in neurons as in the brain (Montagu 1957 Carlsson *et al* 1962 Fuxe 1965) or in the heart (Angelakos *et al* 1963) Of particular interest is the finding of up to 60 per cent DA of the total catecholamines in the glomus cells of the carotid body in the cat (Chiocchio *et al* 1967) which has been confirmed also for the human carotid body tumour (Lishajko *to be published*)

Recently Woodruff and Walker (1969) have reported the presence of a specific DA receptor in the brain of the snail, mediating inhibition of the spontaneous activity of the neurons The effect was associated with hyperpolarization of the cell membrane on which NA and A were 25 respectively 90 times less potent

The present findings raise the question as to whether DA may exert physiological effects in connection with the secretion of NA and A Whether such effects occur on the vascular system (*cf* Goldberg and Sjoerdsma 1959) or affect the secretory process by an action on the transmission or else on the cell membrane (*cf* Woodruff and Walker 1969) cannot at present be decided

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References

ANGELAKOS E T K FUXE and M L TORCHIANA Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts *Acta physiol scand* 1963 *59* 184—192

BERTLER A B FALCK N A HILLARP E ROSENGREN and A TORP Dopamine and chromaffin cells *Acta physiol scand* 1959 *47* 251—258

BERTLER A N A H [illegible] intestine *Acta phys* [illegible]

BUTTERWORTH K R [illegible] *content of the left* [illegible] 299

CARLSSON A and B WALDECK A fluorimetric method for the determination of dopamine (3 hydroxytyramine) *Acta physiol scand* 1958 *44* 293—298

CARLSSON A B FALCK and N A HILLARP Cellular localization of brain monoamines *Acta physiol scand* 1962 *56* Suppl 196
CHIOCCHIO S R A M BISCARDI and J H TRAMEZZANI 5 Hydroxytryptamine in the carotid body of the cat *Science* 1967 *158* 790
DOUGLAS W W and R P RUBIN The role of calcium in the secretory response of the adrenal medulla to acetylcholine *J Physiol* (Lond) 1961 *159* 40—57
DOUGLAS W W and R P RUBIN The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling *J Physiol* (Lond) 1963 *16* 288—310
EADE N R The distribution of the catecholamines in homogenates of the bovine adrenal medulla *J Physiol* (Lond) 1958 *141* 183—192
EULER U S v and F LISHAJKO Dopamine in mammalian lung and spleen *Acta physiol pharmacol neerl* 1957 *6* 295—303
FALCK B N A HILLARP and A TORP A new type of chromaffin cells probably storing dopamine *Nature* (Lond) 1959a *183* 267—268
FALCK B N A HILLARP and A TORP, Some observations on the histology and histochemistry of the chromaffin cells probably storing dopamine *J Histochem Cytochem* 1959b *7* 323—328
FUXE K The distribution of monoamine nerve terminals in the central nervous system *Acta physiol scand* 1965 *64* Suppl 247
GOLDBERG L I and A SJOERDSMA Effects of several monoamine oxidase inhibitors on the cardiovascular actions of naturally occurring amines in the dog *J Pharmacol exp Th* 1959 *127* 212—218
GOODALL McC Studies of adrenaline and noradrenaline in mammalian heart and suprarena *Acta physiol scand* 1951 *24* Suppl 58
HEMPEL K and H F K MANNL Resting secretion of dopamine from the adrenal glands the cat in vivo *Experientia* (Basel) 1967 *23* 919
HEMPEL K and H F K MANNL Quantitative Analyse der Catecholamin Biosynthese Nebennierenmarks in vivo und Ruhesekretion neugebildeter Amine unter besonderer rucksichtigung des Dopamins *Naunyn Schmiedeberg s Arch exp Path Pharmak* 1969 *2* 363—388
KIRSHNER N H J SAGE W J SMITH and A G KIRSHNER Release of catecholamines a specific protein from adrenal glands *Science* 1969 *154* 529—531
LISHAJKO F Occurrence and some properties of dopamine containing granules in the she adrenal *Acta physiol scand* 1968 *72* 255—256
LISHAJKO F Release reuptake and net uptake of dopamine noradrenaline and adrenaline isolated sheep adrenal medullary granules *Acta physiol scand* 1969 *76* 159—171
LISHAJKO F (to be published)
MONTAGU K A Catechol compounds in rat tissues and in brains of different animals *Natu* (Lond) 1957 *180* 244—245
PHILIPPU A and H J SCHUMANN Der Einfluss von Calcium auf die Brenzcatechinaminfr setzung *Experientia* (Basel 1962 *18* 138—140
PUPPI A I BENEDECZKY and A TIGYI Identification of dopamine containing granules in t adrenal medulla *Acta physiol Acad Sci hung* 1965 *27* 341—347
SHEPHERD D M and G B WEST Hydroxytyramine and the renal medulla *J Physi* (Lond) 1953 *120* 15—19
STJARNE L Studies of catecholamine uptake storage and release mechanisms *Acta physi scand* 1964 *62* Suppl 228
WOODRUFF G N and R J WALKER The effect of dopamine and other compounds on t activity of neurons of *Helix aspersa* structure activity relationships *Int J Neuropharm* 1969 *8* 279—289

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Bretylium and the Degeneration Contraction of the Sympathetically Innervated Periorbital Smooth Muscle in the Rat

By

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Abstract

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The ability of bretylium to delay the start of the degeneration contraction of the sympathetically innervated periorbital muscle has been studied in conscious rats. The sympathomimetic and neuron blocking action of bretylium at the doses most frequently used were also tested. A low dose, 0.5 mg/kg, that induced no measurable neuron block still delayed. The delay caused by local injection into the muscle exceeded the duration of the neuron block. These findings indicate that the bretylium induced delay is not caused by neuron block and that the delaying action mainly is exerted on the terminal parts of the neurons. Bretylium given repeatedly postponed the onset of the degeneration contraction at least 6 to 10 hrs. Later the processes [illegible] transmitter release. It appeared that early during the degeneration contraction there are nerve endings at different stages of degeneration on which bretylium acts differently.

The ability of bretylium to delay the postdenervation disappearance of noradrenaline from sympathetically innervated organs in the rat was first reported by Benmiloud and Euler (1963). Later it was verified histochemically by Malmfors and Sachs (1965). In 1966 Langer reported that β-TM10, which is closely related to bretylium pharmacologically, delayed the degeneration contraction in the nictitating membrane of the cat. This denervation phenomenon and other similar spontaneous postdenervation excitatory effects are thought to be related to the leakage of transmitter from the degenerating adrenergic nerve terminals (Sears and Bárány 1960, Coats and Emmelin 1962, Langer 1966, Lundberg 1969). In a recent study of the degeneration contraction of the sympathetically innervated periorbital smooth muscle in the rat, bretylium was compared to the neuron blockers β-TM

bethanidine and BW 392C60 (Lundberg 1969). They were all less potent than bretylium in delaying the onset of the degeneration contraction

The present paper deals with further studies on the ability of bretylium to influence the time course of the degeneration contraction in the rat

Materials and methods

Experimental animals

Male Sprague-Dawley rats weighing about 250 g were used They were kept in normal day light conditions at around 23° C Commercial rat food pellets (No 210, Anticimex, Sollentuna, Sweden) and tap water were provided *ad lib*

Surgical procedure

The right superior cervical ganglion was removed (denervation) and the left pre ganglionic trunk was cut (decentralisation) under ether, except in 1 expt in which both sides were denervated

Drugs

Bretylium tosylate was kindly donated by Dr A F Green, the Wellcome Research Laboratories, Beckenham, Kent The drug was dissolved in 0 9 % NaCl just before use and injected s c if not otherwise stated Doses refer to the salt

Measurement procedure

The sizes of the palpebral aperture of the two eyes in conscious animals were measured at a distance every 1 to 1 1/2 hrs during the period of study except in connection with the injections Then they were measured just before the injection and about 1/2 hr later A special apparatus with an image splitting eyepiece was used The difference in mean size of aperture between the denervated and the decentralized (control) side on every occasion of measurement was plotted against the time after denervation Curves of individual animals thus obtained were then analyzed as earlier described (Lundberg 1969) The degeneration contraction was characterized from the following points of view

The time course Usually the times corresponding to 50 % of maximal difference between denervated and decentralized side on the ascending part (T_{50a}) and on the descending part (T_{50d}) of the curves were used to describe the time course In some experiments, however, the curves had two humps When such curves have to be compared either between themselves or ... es In these series therefore the times corresponding ... m on the first ascending limb (T_{10a}) and on the ... were used instead of T_{50a} and T_{50d} respectively ... about 50 % of the peak difference of a normal degeneration contraction In some groups T_{50d} or T_{10d} values were not obtained in all rats because the experiment would have lasted too long Then the median or the possible range of the median value was calculated instead of the mean

The duration (width) This was measured as the difference between T_{50d} and T_{50a} or between T_{10d} and T_{10a}

The height This was the maximal value of palpebral aperture measured on the denervated side during the degeneration contraction, no regard being taken to the control side

Estimation of the sympathomimetic effect

The immediate increase in palpebral aperture following an injection of bretylium has been considered as a sympathomimetic effect of the drug With the doses used this effect always reached its maximum within 1 hr after the subcutaneous injection of bretylium and lasted only for 2 to 4 hrs The magnitude of the effect was estimated by measuring the difference between the value noted at 1 hr after the injection and the average of 3 to 5 consecutive values taken from the flat part following the peak of the individual curve The first occasion when the curve had reached its flat base was taken as the end of the sympathomimetic effect

Estimation of the neuron blocking effect

The ptosis induced by bretylium in rats with intact sympathetic innervation is most likely a neuron blocking effect Its magnitude was estimated as the difference between the lowest value of the palpebral aperture noted and the pretreatment value As parameters of duration were

[illegible]

S E M are shown

Time of injection (hrs after den)	Number of rats with equal (E) or unequal (U) palpebral apertures		Increase in palpebral aperture at 1 hr after injec ion (mm)	
	E	U	Denervated side	Decentralized side
10	5	—	0 14±0 12	0 14±0 12
11	10	—	0 35±0 08	0 35±0 08
12	9	—	0 24±0 08	0 24±0 08
12	—	3	0 71 0 45 0 52	0 03 0 00 0 25
13	3	—	0 35 0 19 0 01	0 35 0 19 0 01
13	—	2	0 86 0 73	0 26 0 20
			←p<0 01→	
14	—	5	0 81±0 15	0 14±0 10

used either the time after injection needed for 50 % of the maximal effect to disappear or the time of the first occasion after the injection with a value equal to or higher than the pretreatment value

Student s t test was used for the analysis of significance

Results

The sympathomimetic effect of bretylium given at different times after denervation

The sympathomimetic effect has been measured incidentally in experiments primarily designed to study the delaying effect of bretylium and reported earlier (Lundberg 1970) or in this paper The excitatory effect of bretylium at the doses most frequently used was small and comparatively short Because of this and since the apertures in most cases were measured only at hourly intervals the estimations of the sympathomimetic effects were quite rough The results are shown in Table I With 4 mg/kg given at 10 or 11 hrs after denervation the increase in palpebral aperture was symmetric and small This was so even in 9 of the 12 rats treated at 12 hrs and in 3 of the 5 rats given the drug at 13 hrs after denervation However, in the other 12 or 13 hrs rats and always in the 14 hrs group the sympathomimetic effect of bretylium tended to be asymmetric with a stronger effect on the denervated side than on the decentralized side The duration of the excitatory effect did not exceed 4 hrs in any case The peak effect was observed within or at 1 hr after the injection The results

suggest that the conditions for a sympathomimetic effect of bretylium change during the 4 hrs which precede the start of the normal degeneration contraction

Neuron blocking effect of bretylium at doses used in the studies of the delaying action

a Bretylium given subcutaneously

4 rats with intact sympathetic innervation were injected with bretylium at 4 mg/kg The palpebral apertures were measured at intervals of 1/2 to 1 hr The maximal decrease in aperture *i e* the maximal degree of ptosis was 1 42 ± 0 29 mm (mean and S E M) and was measured 1 to 2 hrs after the injection Fifty per cent of the peak effect had disappeared 2 7 ± 0 2 hrs after the injection and the total duration did not exceed 4 hrs in any case In 5 normal rats treated with 0 5 mg/kg of bretylium and in a similar group given 0 9 % NaCl there were no measurable ptosis Since 0 5 mg/kg of bretylium given at 11 hrs after denervation shifts the start of the degeneration contraction from about 4 hrs to 8 hrs after the injection (Lundberg 1970) there seems to be no simple relation between the delaying and the neuron blocking actions of the drug However the possibility remains that the degenerating nerve treats bretylium differently

b Bretylium given locally into the periorbital tissue

5 normal rats were lightly anesthetized with ether and given bretylium at 0 08 mg/kg into the lower lid and the muscular floor of the orbit on one side On the other side an equal volume of the solvent (0 1 ml/kg) was injected The solvent was 0 9 % NaCl with 10 I E per ml of testicular hyaluronidase (Hyalas® Leo Halsingborg Sweden) The injection itself caused no change in the aperture at 30 min The palpebral apertures were estimated at 1/2 hr intervals after the injection On the control side there was very small decrease in size or no change at all The maximal decrease in size of aperture on the treated side 0 87 ± 0 11 mm was measured within the first hr after the injection Fifty per cent of the effect had disappeared 1 5 ± 0 2 hrs after the injection In no case the difference between the two sides persisted for more than 2 5 hrs

Delaying effect of bretylium injected locally

5 rats were denervated by ganglionectomy on both sides simultaneously 11 hrs after the denervation the animals were treated locally with 0 08 mg/kg bretylium as described above This dose of bretylium gives no systemic effects (Lundberg 1970) and the neuron blocking effect should have come to an end at 13 5 hrs The results are shown in *Table II* They were obtained by comparing each eye with its own 3 to 4 pretreatment values On the control side the degeneration contraction reached 50 % of its peak value at the normal time around 15 hrs while on the bretylium treated side this happened about 2 5 hrs later The shape of the contraction curve

TABLE II

Treatment design	Time of start ($T_{10\%}$) hrs[1]	Duration ($T_{10\%}$—$T_{10\%}$) hrs	Height (peak aperture on denervated side) mm
0 08 mg/kg bretylium locally n=5	17 71±0 21 ↑ p<0 005 ↓	5 60—7 90[2]	4 27±0 12
NaCl locally n=5	15 27±0 45	7 89±1 01	4 24±0 13
0 08 mg/kg bretylium systemically n=5	15 44±0 32	7 70±0 51	4 27±0 085
Untreated rats n=70	15 14±0 11	8 03±0 17	4 46±0 051

[1] time after denervation
[2] range of the median

was normal in all the NaCl treated eyes However for practical reasons the contractions on the bretylium treated sides could not be followed to the ends but as long as they were studied, i e 3 5 to 6 hrs after the peaks their appearances seemed to be normal It is evident that the delaying action of bretylium is exerted on the peripheral parts of the nerve The finding also seems to exclude the possibility that the bretylium induced delay is caused by a neuron block stopping release due to an irritative lesion at the cut end of the nerve see Discussion

Effect of bretylium given at different time intervals after denervation

a Bretylium injected when the degeneration contraction has started

Two groups of 5 rats each were injected with single doses of bretylium at 4 mg/kg when the degeneration contraction had been going on for about 1 5 hrs and had reached at least 50 % of its expected peak value The course of every contraction in both groups was interrupted for several hrs by the injection of bretylium In the rats of one group which were denervated in the evening the degeneration contractions did not start again until about 29 hrs after denervation For practical reasons they could not be followed long enough for reliable peak values of the delayed part of the contractions to be obtained Therefore another group of rats was run which was ganglionectomized in the morning The mean curve of the morning group is shown in Fig 1 During the first 1/2 hr after the injection the contraction

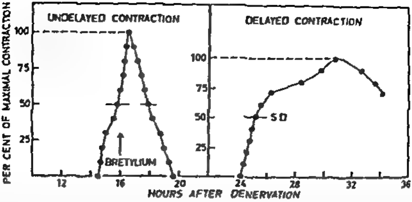

Fig 1 Effect of bretylium given after the start of the degeneration contraction The rats (n= 5) were denervated in the morning Bretylium (4 mg/kg s c) was given at about 16 hrs after denervation The drug interrupted the ongoing contraction and displaced the latter part Mean curves for the two parts of the degeneration contraction are shown S D =standard deviation For the method of construction of mean curves, see Lundberg 1969

on the denervated side seemed to be increased by bretylium This was most easily seen in the individual curves Then the degeneration contraction began to cease and had completely disappeared 2 to 5 hrs later It reappeared after another time interval of 3 to 6 hrs There was a suggestion of a "double hump" in the delayed part of the degeneration contraction The undelayed contraction of the "evening" group had similar shape and size as that of the "morning" group, see Table III However, the delayed contraction of the "evening" group started significantly later than that of the other group The difference between the two groups in onset of the delayed contraction looks as if the contraction of the "evening" group lacked the first part of "double hump" However, this is hard to state definitely because of insufficient data on the delayed part of the "evening" group

b Bretylium injected before the degeneration contraction or at its start

Rats in groups of 5 or 6 were treated with 4 mg/kg of bretylium in single doses at different times ranging from 6 to 14 hrs after denervation. 2 or 3 similar groups per time of injection were run on different days 2 or 6 weeks apart However in the 6 hr group all rats were run simultaneously In all cases the degeneration contraction was delayed Two different types of curves were observed, one curve with smooth appearance similar to that of a normal degeneration contraction and one with a "double hump" Typical "single hump" and "double hump" curves are shown in Fig 2 As explained under "Methods" T_{50_2} and T_{50_d} can be misleading when such curves are to be compared and T_{10_2} and T_{10_d} were used instead in certain comparisons Table IV shows the results In rats given bretylium at 6, 10 or 11 hrs after ganglionectomy all curves were of the normal single hump type and the durations of the contractions were almost normal They were delayed by 3 to 5 hrs However, all the rats of the

TABLE III Effect of bretylium (4 mg/kg s c) given after the start of the degeneration contraction The drug interrupted the ongoing contraction and displaced the latter part, see also Fig 1 T_{sa} is the time of the last measurement before the onset of the contraction The time of half maximum development of the contraction is called T_{50a} and the corresponding value on the descending phase is T_{50d} The values are means ± S E M if not otherwise stated There were 5 rats in each group

Experimental design	Undelayed contraction			Delayed contraction			
	Time of start (T_{50a})	Duration (T_{50d}—T_{50a})	Height (peak aperture on den side)	Time of start (T_{sa})	Time of start (T_{50a})	Duration (T_{50d}—T_{50a})	Height (peak aperture on den side)
	hrs[1]	hrs	mm	hrs[1]	hrs[1]	hrs	mm
Denervated in the evening Injected at 15 80±0 22 hrs[1]	15 36± 0 45	2 30± 0 36	4 45± 0 24	28 69± 0 46	—[2]	—[2]	—[2]
				p<0 001			
Denervated in the morning Injected at 16 [illegible]±0 14 hrs[1]	15 78± 0 32	2 09± 0 16	4 67± 0 12	24 19± 0 28	23 27± 0 36	8 35— 11 00[3]	3 93± 0 23

[1] hrs after denervation
[2] peak not reached during time of observation
[3] range of the median

12 hrs group and 4 out of the 10 rats treated at 14 hrs had lengthened contractions with double humps The contractions of the 13 hrs group had no clear-cut double humps but distinctly increased durations which might indicate a tendency to division of the degeneration contraction into two main parts even in this group If bretylium was given at 14 hrs instead of 13 hrs the delay was very much increased The increase in delay caused by this 1 hr step was about 3 hrs in the 'double hump" and 8 5 hrs in the "single hump' group In 5 out of the 10 rats treated at 14 hrs the contraction was going on at the time of injection It had started less than 1 hr before the

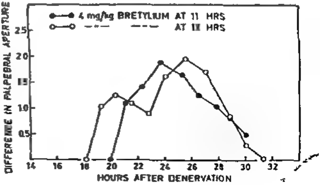

Fig [illegible] Effect of bretylium (4 mg/kg s c) given at 11 or 12 hrs after denervation on the time course of the degeneration contraction Two representative individual curves are shown A normal degeneration contraction would have started at about 14 hrs after denervation and have a smooth appearance

TABLE IV Influence of the time interval between the denervation and the injection on the time course of the degeneration contraction delayed by bretylium (4 mg/kg s c) There were two kinds of contraction curves, single hump and double hump $T_{1\,sa}$ is the time of

curve was broader and flatter than seen in a normal contraction

Time of injection hrs[1]	n	Time of start ($T_{1\,sa}$) hrs[1] and *Delay* hrs	Time of end ($T_{1\,ed}$) hrs[1]	Duration ($T_{1\,ed}$—$T_{1\,sa}$) hrs	Type of curve
6	5	18 70±0 50 *3 30*	25 14±0 68	6 44±0 83	single hump
10	10	19 16±0 37 *3 76*	27 30±0 42	p<0 05 8 14±0 54	—, —
11	10	20 52±0 23 *5 12*	27 62±0 48	7 10±0 39	— —
12	12	*19 82±0 38* *4 42*	*30 14±0 45*	p<0 001 *10 32±0 62*	*double hump*
13	10	20 86±0 36 *5 46*	30 25—32 05[2]	9 45 – 11 25[2]	single (?) hump
14	4	24 15±0 51 *8 75*	> 32 90[3]	~ 9 05[3]	double hump
14	6	29 62±0 51 *14 22*	> 31 10[3]	> 1 45[3]	(?)
untreated rats	21	15 40±0 24	21 98±0 36	6 59±0 36	single hump

[1] hrs after denervation
[2] range of the median
[3] median

injection and had advanced to less than about 40 % of a normal peak value This was so in 1 of the 4 double humps and in 4 of the 6 single humps' These findings indicate that during the time interval 11 to 14 hrs after denervation which precedes or includes the very first part of the normal degeneration contraction the conditions for the effect of bretylium change quickly

Effect of repeated injections of bretylium

5 rats were injected with 4 mg/kg of bretylium every 4 hrs from 13 to 33 hrs after denervation Each rat thus received 24 mg/kg in all Readings in connection with injections were always made immediately preceding the latter The results are shown in Fig 3 No typical degeneration contraction was seen during the time of observation with the possible exception of one case (shown uppermost in Fig 3) Following al

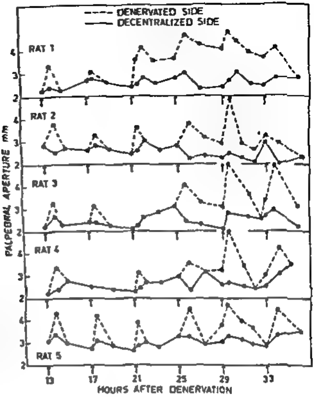

Fig 3 Effect of repeated injections of bretylium on the degeneration contraction Bretylium (4 mg/kg s c) was given every 4 hrs from 13 to 33 hrs after denervation The injections are indicated by arrows Readings in connection with injections were always made immediately preceding the latter

most every injection there was an asymmetric sympathomimetic effect On the decentralized side the post-injectional widening of the palpebral aperture did not change consistently during the observation time On the denervated side it was small and did not last for more than 2 hrs after the first injections From 25 hrs after denervation and later on, however, it was distinctly increased and prolonged At 29 hrs the palpebral aperture in all cases was wider on the denervated than on the decentralized side and in two cases (rat 4 and 5 in Fig 3) the apertures on the denervated side had spontaneously increased in size since the latest occasion of measurement *Thus, with repeated injections of bretylium the degeneration contraction generally is* delayed at least until about 25 hrs after denervation At this time the conditions seem to change

Discussion

Spontaneous excitatory effects following denervation, such as the degeneration contraction studied in this paper, are most probably functional responses to the degeneration transmitter release for ref see Introduction The way of action by which bretylium delays the degeneration contraction is not known There seems to be no

simple relation between the neuron blocking and the delaying action of the drug (Lundberg 1969) This view is also favoured by the present finding that bretylium at 0 5 mg/kg which is known distinctly to delay the start of the degeneration contraction (Lundberg 1970), lacked neuron blocking activity on intact sympathetic nerves Similar conclusions can be drawn from the local injections Bretylium at 0 08 mg/kg injected locally into periorbital tissues with intact adrenergic innervation was shown to cause a neuron block of less than 2 5 hrs duration A similar unilateral injection at 11 hrs after denervation shifted the start of the degeneration contraction on the treated side from about 4 hrs to 6 5 hrs after the injection Since the neuron blocking effect of the drug ought to be ended 2 5 hrs after the injection this finding seems to exclude that bretylium delays by acting as a neuron blocker that stops release due to *e g* an irritative lesion at the cut end of the nerve Admittedly, however, one can still not exclude the possibility that the degenerating nerve endings react differently to bretylium and that its neuron blocking action is much prolonged under these circumstances

There was no delay on the control side in the experiment with the local injections This fact indicates that the delaying action of bretylium is mainly exerted on peripheral parts of the neuron and is not due to interference by bretylium with the systems of intra axonal transport which might well be related to the processes startin the degeneration transmitter release

Bretylium is selectively accumulated in adrenergic nerves (Boura *et al* 1960) an not metabolized (Duncombe and McCoubrey 1960) Based on indirect evidence i has been proposed that the onset of the degeneration contraction delayed by bretylium given at 11 hrs after denervation is related to the rate of the disappearance o the drug from the nerve endings (Lundberg 1970) In the present study it wa found that in the hours around the normal start of the contraction the delaying ef fect of an injection of bretylium increased very much This finding suggests that the conditions for uptake and retention of bretylium are changing around this time May be the drug reaches critical structures in the degenerating nerve terminals more easily around the start of the degeneration contraction

There are other findings in the present paper which are difficult to interpret Bretylium given at 12 hrs after denervation or later sometimes caused a *double hump* in the degeneration contraction Even the first hump was delayed but the second was more so This two phase phenomenon which is not seen normally or when bretylium is given earlier also points to a change in the conditions for the action of bretylium during the course of the degeneration Malmfors and Sachs (1965) studied the degeneration depletion of sympathetic transmitter from the rat iris histochemically Based mainly on the lack of transitional stages between normal and degenerate terminals in preparations *taken 4 hrs apart they concluded that* the terminals at some point of time—which differs from system to system—lose their transmitter content quite suddenly (probably within 1 or at the most 2 hrs) Thus at a certain point of time during the course of the degeneration there are systems of nerve endings with different remaining life spans The double hump could be due to the

fact that at the time of injection certain neurons have reached a critical stage where the effect of bretylium is less This set could be either a more or less random part of the population of nerve terminal systems or include the innervation of one of the main portions of the periorbital muscle located at a different distance from the cut end of the post ganglionic nerve

Bretylium was shown to interrupt the ongoing degeneration contraction completely for several hours This indicates that during the first part of the contraction there are still bretylium sensitive nerve endings left Before the interruption the contraction was augmented for about 1/2 hr after the injection The excitatory effect was asymmetric with no appreciable effect on the decentralized side Since the sympathomimetic action of bretylium normally is thought to be due to release of transmitter from the nerve endings (Boura and Green 1959 Green 1962) the asymmetric sympathomimetic effect found in this study could be related to increased release of transmitter (perhaps partly due to inhibition of monoamine oxidase) on the denervated side but also to beginning supersensitivity (loss of axonal amine pump) This would be analogous to the sympathomimetic action of tyramine which has been reported to be increased around the start of the degeneration contraction of the nictitating membrane of the cat (Fleckenstein and Burn 1953 Langer and Trendelenburg 1966) The latter authors showed that this effect of tyramine was related both to increased release of noradrenaline and to increased sensitivity to the released transmitter Simultaneously with the development of the postdenervation depletion of the stores of the adrenergic transmitter in the rat iris the uptake of amines by the axonal amine pump diminishes (Malmfors and Sachs 1965) Similar findings were reported by Smith *et al* (1966) from studies on the degenerating nictitating membrane of the cat This means that the sensitivity to the transmitter released to the vicinity of the degenerating nerve terminals may gradually increase during the course of the degeneration contraction The present experiments however do not allow an assessment of the relative importance of increased release and supersensitivity respectively

It is of interest to know for how long bretylium can delay the onset of the degeneration contraction The present experiment with repeated injections of bretylium unfortunately gives no clear cut information about the maximum delay However some observations seem to be of interest First there was no typical degeneration contraction with the possible exception of one case Second in connection with almost every injection there was an asymmetric sympathomimetic effect which was distinctly increased in connection with injections given later than 25 hrs after denervation Third at 29 hrs in ? of the 5 rats the degree of contraction had spontaneously increased since the previous measurement 1 hr earlier Thus the conditions seem to be changed after 25 hrs probably due to progressing bretylium insensitive degeneration processes These findings may also fit the hypothesis that there are systems of nerve endings at different stages of degeneration on which bretylium acts differently In most cases the axons seemed to be bretylium sensitive up to about 25 hrs after the denervation La er the processes responsible for the degeneration leakage

develop despite the treatment Therefore, the initially bretylium sensitive systems of nerve terminals change into intermediate and advanced stages of degeneration in which the release may be precipitated by bretylium or occurs spontaneously

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References

Benmiloud M and U S v Euler Effects of bretylium reserpine guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland *Acta physiol scand* 1963 *59* 34—42

Boura A L A F C Copp W G Duncombe A F Green and A McCoubrey The selective accumulation of bretylium in sympathetic ganglia and their postganglionic nerves *Brit J Pharmacol* 1960 *15* 265—270

Boura A L A and A F Green The actions of bretylium adrenergic neurone blocking and other effects *Brit J Pharmacol* 1959 *14* 536—518

Coats D A and N Emmelin The short term effects of sympathetic ganglionectomy on the cat's salivary secretion *J Physiol (Lond)* 1962 *162* 282—288

Duncombe W G and A McCoubrey The excretion and stability to metabolism of bretylium *Brit J Pharmacol* 1960 *15* 260—264

Fleckenstein A and J H Burn The effect of denervation on the action of sympathetic amines on the nictitating membrane *Brit J Pharmacol* 1953 *8* 69—78

Green A F Antihypertensive Drugs *Advances in Pharmacology* 1962 *1* 161—225

Langer S [illegible] The degeneration contraction of the nictitating membrane in the unanesthetized cat *J Pharmacol exp Ther* 1966 *151* 66—72

Langer S [illegible] and U Trendelenburg The onset of denervation supersensitivity *J Pharmacol exp Ther* 1966 *151* 73—86

Lundberg D Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat *Acta physiol scand* 1969 *75* 415—426

Lundberg D Interactions between inhibitors of the axonal amine pump and bretylium in the degeneration release of sympathetic transmitter *Acta physiol scand* 1970 *78* 503—521

Malmfors T and C Sachs Direct studies on the disappearance of the transmitter and changes in the uptake storage mechanism of degenerating adrenergic nerves *Acta physiol scand* 1965 *64* 211—223

Sears M L and E H Bárány Outflow resistance and adrenergic mechanisms *Arch Ophthalm* 1960 *64* 839—848

Smith C B U Trendelenburg S Z Langer and T H Tsai The relation of retention of norepinephrine H^3 to the norepinephrine content of the nictitating membrane of the spinal cat during development of denervation supersensitivity *J Pharmacol Exp Ther* 1966 *151* 87—94

Acta physiol. scand. 1970. 79. 423—430

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Steps in Impulse Generation in the Isolated Muscle Spindle

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Abstract

OTTOSON, D. and G. M. SHEPHERD. *Steps in impulse generation in the isolated muscle spindle.* Acta physiol. scand. 1970. 79. 423—430.

[illegible] rectly related to the rate of rise of the prepotential. A similar relationship was found for the discharge frequency and the amplitude of the receptor potential. The results thus show that there is a close interrelationship between the three main stages of impulse generation. This finding explains the ability of the spindle to pass on faithfully, in the frequency characteristics of the impulse discharge, information about the properties of the applied stretch.

It is generally recognized that in their response to a stimulus sensory receptors produce a graded generator potential which triggers the nerve impulse. However, the receptor potential does not directly lead to the initiation of the conducted activity, interposed between the two events is the development of the prepotential leading up to generation of an impulse. There are thus three stages in the sequence of electrical processes leading to production of the impulse discharge. Each of these steps must be important in determining the frequency characteristics of the impulse response of the afferent fibre. The relation between the receptor potential and the impulse frequency has been established in studies of the frog muscle spindle (Katz 1950 *a*, *b*, Ottoson and Shepherd 1965) and on the crustacean stretch receptor (Terzuolo and Washizu 1962). There is, however, at present little information as to the quantitative relationship between these two events and the prepotentials of the nerve.

The aim of the present study was to study the quantitative relationship between the transducer action of the muscle spindle, as represented by the receptor potential, the prepotentials and the impulse discharge of the afferent nerve fibre during various phases of stretch. The experiments were carried out on isolated frog spindles which

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were subjected to controlled linearly rising stretches It was found that the rate of rise of the prepotentials closely followed the changes in depolarization of the sensory endings, similarly the impulse frequency was closely related to the properties of the prepotentials The regular interrelationship between the three main stages of impulse generation appears to explain the ability of the spindle to transmit information about the characteristics of the stimulus with a high degree of precision

Methods

[illegible]

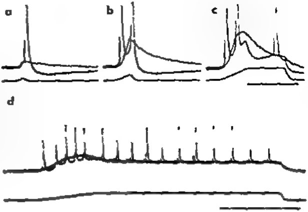
details are given in Shepherd and Ottoson (1965) and Ottoson McReynolds and Shepherd (1969)

Results

Receptor potential/impulse frequency relationship The response recorded from the afferent nerve of the spindle at its issuance from the spindle capsule is composed of the receptor potential the local responses preceding the impulses and the propagated impulses The receptor potential can be obtained in isolation by blocking the impulse activity of the nerve with local anesthetics (Katz 1950 b) By subjecting the spindle to a series of stretches before and after blockage, the contribution of the graded potentials of the sensory endings to the composite response can thus be analyzed The method applied in the present study is illustrated in Fig 1 Records *a—c* show the responses elicited by stretching the spindle at constant velocity to three different lengths, in *c* stretch was maintained at constant length for sufficient time to allow the first static spike to appear The superimposed receptor potentials were obtained with the same stretches after blockage of the impulse activity Record *d* illustrates the relation between the impulse response and the receptor potential during a more prolonged stretch

From recordings like those illustrated in Fig 1 a quantitative relationship between the receptor potential and the impulse frequency of the nerve discharge can be obtained The results of such an analysis are illustrated by the diagram in Fig 2 The frequency of the dynamic discharge during a relatively slowly rising stretch is plotted against the level of the receptor potential at which the individual spikes arose The values for the static discharge were obtained by subjecting the same spindle to sustained stretches at different lengths For both the dynamic and the static discharge the frequency increases linearly with depolarization of the endings For a given amount of depolarization the firing rate is higher during dynamic stretch than during

Fig 1 Relation between impulse response and underlying receptor potential Records obtained before and after bathing spindle in 0 2 % lignocaine *a—c*, response to brief stretches showing development of dynamic impulse response and transitional interval in relation to receptor potential, *d*, similar recording with more slowly rising prolonged stretch Records photographically superimposed Time bar in *c*, 25 msec, in *d*, 100 msec Lower traces show stretch monitor in this and following figures



static stretch The slope of the line for dynamic stretch varied with the rate at which the spindle was stretched while the slope of the static line remained unaffected Since recording was made extracellularly the values in the diagram do not give the absolute amount of depolarization of the endings In order to establish this relationship intracellular recordings would have to be made, which was not possible with the present technique

Receptor potential/prepotential relationship In Fig 3 A is illustrated the response of a spindle to a brief stretch giving rise to a single spike The contribution of the prepotential leading up to the spike is disclosed after blocking the conducted activity with lignocaine It can be seen that the prepotential develops from the receptor potential and increases the rate of depolarization leading to the initiation of the spike With a prolonged stretch the same event is repeated for each impulse

The records in Fig 3 B give an example of the regular change in rate of rise of the prepotential during a slowly rising linear stretch As stretch proceeds gradually to

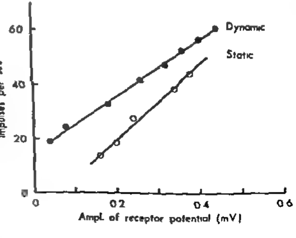


Fig 2 Relation between amplitude of receptor potential and frequency of impulse discharge during dynamic and static phase of stretching Dynamic values obtained from response to linearly increasing stretch at 1 ? mm/sec Static values from the same spindle subjected to steady stretches of different lengths

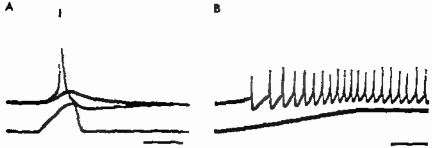


Fig 3 *A* Response to brief stretch before and after bathing the spindle with 0 18 % lignocaine Note that lignocaine blocks the action potential and the small prepotential leading up to the action potential Time 20 msec

B Composite response during slowly rising dynamic stretch Note regular increase in rate of rise of prepotentials as dynamic stretch proceeds Time 100 msec

higher levels the prepotentials rise successively faster and impulse frequency increases as a consequence of this As can be seen, there is also a gradual rise in threshold for the successive spikes during dynamic stretching As demonstrated earlier (Ottoson and Shepherd 1965) the depolarization of the sensory endings increases proportionally with lengthening of the spindle It may be concluded therefore that the rate of growth of the prepotential is determined by the amount of depolarization This is in close accordance with observations on *Carcinus* nerve (Hodgkin 1948) and on the crustacean stretch receptor (Eyzaguirre and Kuffler 1965)

The rate of growth of the prepotential is also influenced by the time course of ... ent of the depolarization spreading from the endings This may be demon ...ited by subjecting the spindle to different velocities of stretch In the experiment ...strated in Fig 4 the spindle was stretched to the same level at three velocities The response appeared with different latencies but for the purpose of illustration the records have been superimposed as if the first spike of each of the responses occurred at the same delay As can be seen the second spike was preceded by a gradually rising potential the rate of growth of which reflected the increase of the prepotential and of the underlying receptor potential which also rose faster with increasing velocity of stretching It may also be noted that with faster stretches the spikes were reduced in amplitude and their level of take-off seemed to rise, these characteristics are possibly related to refractoriness of the nerve (Ottoson and Shepherd 1969) and to the amplitude of the underlying receptor potential

From the above described experiments it may be concluded that the rate of growth of the prepotentials is determined by the amount and rate of increase of depolarization as represented by the receptor potential This conclusion is borne out by an analysis of the quantitative relation between the amplitude of the receptor potential and the rate of rise of the prepotentials As is illustrated in the diagram in Fig 5 *A* the rise of the prepotential becomes faster with increasing amounts of depolarization of the endings during dynamic stretch

Prepotential/impulse frequency relation The regular relation between the rate of

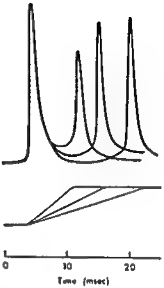


Fig. 4. Change in rate of rise of prepotential leading up to second dynamic spike with change in velocity of dynamic stretch. Tracings from three recordings superimposed so that the first spike appears with the same latency.

growth of the prepotentials and the amount of depolarization of the sensory endings suggests that the frequency characteristics of the response during dynamic stretch are directly determined by the prepotential. This was confirmed by measurements of the frequency of the discharge and the rate of rise of prepotentials during a dynamic stretch, as illustrated in the diagram in Fig. 5 *B*.

At the transition from dynamic to static stretch the frequency in firing falls abruptly from its dynamic peak to a lower level and then declines slowly during static stretch (Shepherd and Ottoson 1965). These changes are related to the fall of the underlying receptor potential from its dynamic peak. Examination of the change in rate of growth of the prepotentials shows that there is a similarly close relation between their slopes and the decline in firing rate. The close relationship between receptor poten

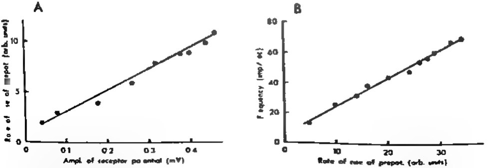


Fig. 5. *A*. Relation between rate of rise of prepotentials and amplitude of receptor potential after treatment of the spindle with 0.18 % lignocaine.
B. Relation between frequency of impulse discharge and rate of rise of prepotentials.

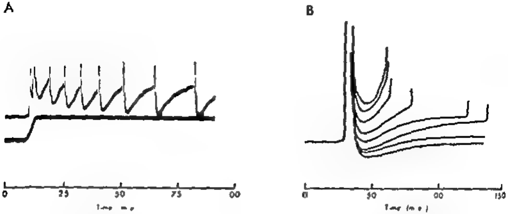


Fig. 6. *A*. Change in rate of rise of prepotential during early phase of static stretch.
B. The effect of increasing stretch on rate of rise of prepotential leading up to first static spike. The initial spike summated in all tracings is the single dynamic spike response to the step stretches.

tial prepotential and spike intervals during the initial period of static stretch is illustrated in Fig. 6 *A*.

In studies of the response of the crustacean stretch receptor, Eyzaguirre and Kuffler (1955) found that the prepotentials increased faster with increasing amounts of stretch. The muscle spindle exhibits closely similar properties as seen in Fig. 6 *B*. The spindle was in this experiment subjected to a steplike stretch to different lengths. The superimposed records show that the prepotential preceding the first static spike was graded with stretch and increased successively faster as stretch was made stronger. As a result of this change the firing level was reached sooner and the spike was elicited earlier. The firing level was not constant; it increased in direct relation to the amount of stretch and consequently the amplitude of underlying receptor potential. Measurements of the rate of rise of the prepotentials for different levels of stretch showed a direct relation to the amplitude of receptor potential and frequency of spike discharge similar to that for the dynamic period of stretch (*cf.* Fig. 5).

Discussion

The present study has provided evidence for steps in the impulse production of the muscle spindle similar to those in the crustacean stretch receptor (Eyzaguirre and Kuffler 1955). In the stretch receptor the potential immediately preceding the impulses was designated a prepotential. This potential included the local response of the nerve as well as the potential changes taking place in the aftermath of a previous impulse. In the present paper the term has been used with a similar meaning. The response to a brief stretch giving a single impulse (*cf.* Fig. 3 A) provides evidence for the local potential alone, and it is assumed that this is part of the prepotential during repetitive activity.

It has been shown in previous studies (*Shepherd and Ottoson* 1965) that the dynamic as well as the static properties of the stretch are closely reproduced by the receptor potential The present results show that the information embodied in the receptor potential ■ preserved during the second step involving production of pre potentials leading up to generation of impulses in the nerve This ■ evidenced by the close relationship between the properties ■f the prepotentials and the receptor poten tial As shown the growth of the prepotentials is directly related to the amplitude and rate of rise of the receptor potential Since the propagated impulses arise when the prepotentials reach threshold level it follows that the rate of rise of the prepotentials is the factor which directly determines the frequency characteristics of the response

The rate of rise of the prepotentials appears to be the outcome of several factors An important factor is the rate of change of the receptor potential this is supported by two lines of evidence The graph of Fig 2 shows that for a given instantaneous amplitude of receptor potential the dynamic impulse frequency is higher than the static frequency This suggests that the rate at which the nerve membrane generates its local response is related to the rate of increase of the background depolarization spreading from the sensory endings This rate sensitivity of the local response gen erating mechanism is in addition to and quite separate from the rate sensitivity of the transduction process in the sensory endings themselves, by which the mechanical stimulus ■ converted into the receptor potential The rate sensitive characteristics of the dynamic impulse discharge are thus dependent on both these factors

Another line of evidence relates to the early period of static stretch when the re ceptor potential decays rapidly from the peak reached at the end of dynamic stretch During this period the ability of the spindle to generate impulses is depressed as shown by the pause that appears between the dynamic and static trains of impulses (Matthews 1931 Katz 1950 *a* Jansen and Matthews 1962 Shepherd and Ottoson 1965) While the absolute level of depolarization during this period is higher than it ■ *later in static stretch* the impulse frequency is lower The present results thus suggest that the rate of development of the prepotentials is also related to the rate of decrease of depolarization or rate of repolarization of the underlying receptor po- *tential The development of the prepotentials and hence the* generation of impulses is depressed during this time and a pause appears in the impulse discharge

In addition ■ the amplitude and rate of change of the receptor potential other *factors may influence the development of the prepotentials One* is refractoriness of the nerve membrane At low rates of discharge the spike intervals last up to 100 msec or more and it may be assumed that the effect of refractoriness is negligible At higher rates of discharge particularly *during dynamic stretch* the spike intervals shorten to 5 msec or less which is approximately the period of relative refractoriness revealed by two shocks to the nerve (Ottoson and Shepherd 1969) If refractoriness is an important factor it must exert its effect in a graded fashion through ■ wide range of impulse intervals because the relation of impulse frequency to different rates and amplitudes of stretch is a linear one (Shepherd and Ottoson 1965) Another factor ■ the threshold level for initiation of impulses which may be expected to vary

with the refractory state of the nerve and the background depolarization of the receptor potential. A final factor to consider is the site of impulse initiation; it is possible that this may vary, being for example nearer the sensory terminals for low levels of receptor depolarization and shifting toward the large nerve branches and stem fibre for large depolarizations spreading from the sensory terminals.

In conclusion, this study identifies an important step in the response of the muscle spindle, that of the generation of prepotentials. It has been shown that the rate of rise of the prepotentials is directly correlated with properties of the receptor potential on the one hand, and frequency of impulse discharge in the stem fibre of the afferent nerve on the other. These correlations are linear despite the fact that the rate of rise of the response is a complex outcome of several factors. By this means the spindle is able to preserve and pass on faithfully, in the frequency of its impulse discharge, information about the parameters of an applied stretch.

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References

EYZAGUIRRE, C. and S. W. KUFFLER: Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. *J. gen. Physiol.* 1955, *39*, 87—119.

HODGKIN, A. L.: The local electric changes associated with repetitive action in a non-medullated axon. *J. Physiol. (Lond.)* 1948, *107*, 165—181.

JANSEN, J. K. S. and P. B. C. MATTHEWS: The central control of the dynamic response of muscle spindle receptors. *J. Physiol. (Lond.)* 1962, *161*, 357—378.

KATZ, B.: Action potentials from a sensory nerve ending. *J. Physiol. (Lond.)* 1950 a, *111*, 248—260.

KATZ, B.: Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol. (Lond.)* 1950 b, *111*, 261—282.

MATTHEWS, B. H. C.: The response of a single end organ. *J. Physiol. (Lond.)* 1931, *71*, 64—110.

OTTOSON, D., J. S. MCREYNOLDS and G. M. SHEPHERD: Sensitivity of isolated frog muscle spindle during and after stretching. *J. Neurophysiol.* 1969, *32*, 24—34.

OTTOSON, D. and G. M. SHEPHERD: Receptor potentials and impulse generation in the isolated [illegible] 1965, *30*, 105—114.

O[illegible] to impulse genera[illegible]

TE[illegible] generator potential and impulse frequency in stretch receptor of crustacea. *J. Neurophysiol.* 1962, *25*, 56—66.

SHEPHERD, G. M. and D. OTTOSON: Response of the isolated muscle spindle to different rates of stretching. *Cold Spr. Harb. Symp. quant. Biol.* 1965, *30*, 95—103.

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From the University Laboratory of Physiology, Oxford, England

A Reply to Criticism of the Hypothesis That the Group II Afferents Contribute Excitation to the Stretch Reflex

By

P B C Matthews

In a short theoretical note Grillner (1970) has challenged the interpretation of certain experiments which led to the suggestion that the spindle group II afferents contribute excitation to the tonic stretch reflex of the decerebrate cat (Matthews 1969). In essence, the original experiments compared the strength of the stretch reflex elicited by stretches of graded size with the strength of the reflex elicited by vibrating the same muscle at various frequencies On the twin assumptions that the vibration was a reasonably selective stimulus for the Ia fibres and that it could "drive" them to discharge one impulse per cycle of vibration the stretch reflex then appeared to be unduly strong to arise solely from the Ia fibres It was suggested that the excess of strength of the stretch reflex arose from the spindle secondary endings contributing excitation to it, the secondary endings are excited by stretch but not by vibration This conclusion rested equally on the finding that the two reflex responses did not occlude each other in the way which would be expected if they both depended solely upon the Ia fibres

Grillner (1970), however, denies that the results warrant the conclusion" and implied that no attention had been paid to the well known increase in muscle strength which occurs with increasing muscle length which he apparently felt was sufficient to explain the results without the need for postulating a role for the spindle secondaries In fact the original paper dealt at some length with control experiments devoted to attempting to elucidate the part played by variations of muscle strength in contributing to the results and it was argued that on their own they could not provide an adequate explanation of the findings There is no point in reiterating the description of these control experiments which Grillner has ignored but certain points earlier mentioned in passing can usefully be made more explicit for their significance is perhaps too readily overlooked

First, the reflex experiments were performed with the final length of the muscle, at which the reflexes were measured within a few mm of the maximal length it can take up in the body As Fig 1 shows any variation of muscle strength with muscle length is then rather small for all physiologically important frequencies of discharge

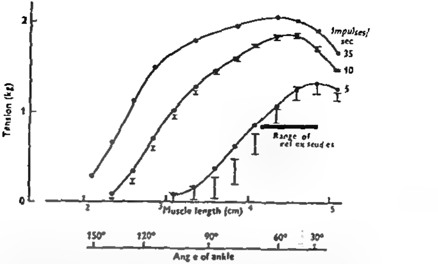

Fig 1 The range of muscle lengths used in the previous reflex studies compared with the tension length curves of the contractions elicited on stimulating the soleus motor fibres at various frequencies The horizontal bar shows the last 7 mm of the physiological range of muscle lengths which was the range in which the reflex responses to stretch and vibration were

is regularly determined in the reflex experi

l*) The tension length curves are taken from the soleus motor fibres in 5 different ventral

circles joined by lines show the results when the stimuli to the different filaments were delivered out of phase with each other so as to give a smooth overall contraction (distributed stimulation) The vertical bars join the peak and ough tensions of the contractions elicited when the various filaments were stimulated in (synchronous stimulation) In both cases each of the individual filaments was stimulated t the frequency shown on the right (The scale of muscle length refers to a slightly arbitrary ero)

Second the amplitude of vibration used (150 μ peak to peak) barely affects the strength of contraction even on low frequency motor activation (Matthews 1966 Joyce Rack and Westbury 1969)

Third the Golgi tendons organs can be expected to be reflexly counteracting the effects of variations of muscle strength They must have been excited by the reflexly induced contractions and by their autogenetic inhibitory action on soleus motoneurones can be presumed to have provided a negative feedback pathway from tension changes to motor discharge in the usual servo manner this should have tended to counteract variation of muscle strength with muscle length by appropriately adjusting the reflex output (Matthews 1959 1967 Houk Singer and Goldman 1969)

Thus the matters emphasized by Grillner do not automatically invalidate the earlier suggestion of an autogenetic excitatory role for the group II fibres and for his objection to be sustained it should be required to deal also with the original control experiments suggesting that variation of muscle strength was not significant under the particular conditions studied However the newly suggested role of the spindle group II fibres should be clearly recognised to be a hypothesis based on in

direct evidence and argument by exclusion. It currently provides the simplest and the only comprehensive explanation for all the observed results, but its validity requires to be tested by further experiment.

References

GRILLNER, S. Is the tonic stretch reflex dependent upon group II excitation? *Acta physiol scand* 1970 *78* 431—432

HOUK, J. C., J. J. SINGER and M. GOLDMAN. Reflex components regulating muscular contraction. In *Proc. of Conf. on Systems Analysis Approach to Neurophysiological Problems* 1969 Minneapolis Univ. of Minnesota.

JOYCE, G. C., P. M. H. RACK and D. R. WESTBURY, The mechanical properties of cat soleus muscle during controlled lengthening and shortening movements. *J. Physiol.* (Lond.) 1969 *204* 461—474

MATTHEWS, P. B. C., The dependence of tension upon extension in the stretch reflex of the soleus muscle of the decerebrate cat. *J. Physiol.* (Lond.) 1959 *147* 521—546

MATTHEWS, P. B. C. The reflex excitation of the soleus muscle of the decerebrate cat caused by vibration applied to its tendon. *J. Physiol.* (Lond.) 1966 *184* 450—472

MATTHEWS, P. B. C., [illegible] *...ibular Mechanisms* 1967 [illegible]

MATTHEWS, P. B. C. [illegible] the muscle spindles in [illegible] at *J. Physiol.* (Lond.) 1969 *204* 365—393

RACK, P. M. H. and D. R. WESTBURY, The effects of length and stimulus rate on tension in the isometric cat soleus muscle. *J. Physiol.* (Lond.) 1969 *204* 443—460

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From the Institute of Neurophysiology, University of Copenhagen, Denmark

Contraction Times and Fibre Types in Intact Human Muscle

By

FRITZ BUCHTHAL AND HENNING SCHMALBRUCH[1]

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Abstract

BUCHTHAL, F and H SCHMALBRUCH *Contraction times and fibre types in intact human muscle* Acta physiol scand 1970 79 435—452

Twitch responses of individual human muscles have been studied in detail only in the hand (Botelho and Cander 1953 Merton 1954 Desmedt 1958, Lambert *et al* 1961, Slomic, Rosenfalck and Buchthal 1968) Since no method has hitherto been available to record the contraction of small bundles of fibres within a human muscle, the contraction time has not been related to the histochemical findings as has been done in cat (for references see Guth 1968 and Henneman and Olson 1965)

It was the aim of the study reported below (i) to develop a method to record the time course of the force developed during twitch in small bundles of fibres in human muscle, (ii) to measure contraction times in different muscles, and (iii) to relate the spectrum of contraction times to histochemical findings (iv) Since the responses of electrically activated bundles originate from fibres of more than one motor unit, they

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were compared with the contraction times of twitches of single motor units activated voluntarily (v) The twitches of fasciculating fibre bundles in normal muscle were studied by the same method A preliminary note has appeared in Nature (Buchthal and Schmalbruch 1969)

Method

1 *General*

To record the time course of twitches of small bundles of fibres, a needle was inserted into a tendon and the displacement of the needle was measured by a small sensitive strain gauge mounted on a holder affixed to the shaft of the needle (described in 2) The measurement of force and the time course of twitches were compared with results obtained by conventional methods a strain gauge at the wrist or first metatarsal and a condenser myograph attached to the thumb (described in section 4)

2 *The mechano electrical transducer to record from the tendon*

i) The strain gauge was a semiconductor about 70 times more sensitive than ordinary wire strain gauges (Kulite New Jersey, type AEP 350—0 90, gauge factor 130 ± 5 %) It had a resistance of 350 Ω a length of 2 5 mm a width of 0 3 mm and a thickness of 0 02 mm In its

isometric twitch displaced the tip of the needle, which in turn caused the deflection of the steel rod measured by the strain gauge

ii) *Static properties* When the needle was 18 mm long displacement of its tip by 4—5 mm (more than the tendon was stretched during a contraction) was within the range of linearity Deviations from linearity did occur during full contraction of large whole muscles due to vertical and lateral movements of the tendon The smallest displacement of the tip of the needle which could be measured reproducibly was 20—50μ the sensitivity of the set up decreasing with increasing free length of the needle

iii) *Dynamic properties* These were examined by the release of a load applied at right angles to the shaft of the needle to produce stepwise displacement of its tip in the tendon The natural frequency was 2200 Hz the logarithmic decrement was 0 4, the ratio between stiffness and equivalent mass was 2×10^{8} sec $^{-2}$ and the ratio between the viscous damping and the equivalent mass was 1600 sec $^{-1}$ Thus step changes in length lasting 2 5 msec or longer were reproduced undistorted

The lower limit of recording was set neither by the noise of the amplifier nor by the strain

the platysma which inserts onto the skin In a number of experiments the interference the ballistocardiogram was diminished by electronic averaging (see p 6)

muscle during

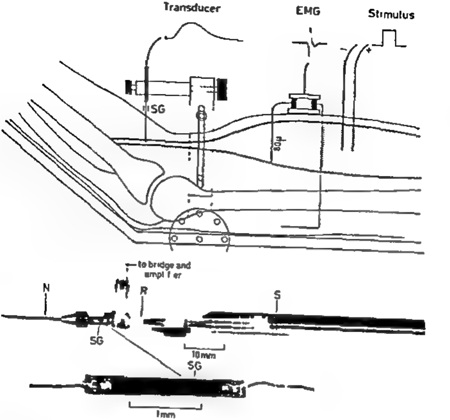

Fig 1 *Abo*...
various po...
The tra...
serted into...
through th... ...ic and a remote electrode or by a concentric electrode ... inserted
Below Mechano-electrical transducer
N needle to be inserted into the tendon
SG semiconductor strain gauge (magnified below) cemented on rod R
S shaft

3 *Activation of small bundles of muscle fibres*

i) *Electrical stimulation in the end plate zone* This method was used in all subjects The electrodes were either two wires each 1 mm long mounted on either side of the shaft of a cannula 0 6 mm in diameter or ... mm apart along the fibres The stimulus ... a doubly screened transformer from a stir... recorded on one channel of the ... mA was ... strength was at or slightly above threshold ... he current

ii) *Electrical stimulation of the nerve* In six subjects the ulnar nerve was stimulated ... the wrist by needle electrod...
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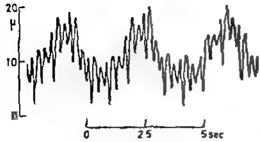


Fig 2 Interference by vibrations corresponding to the "ballistocardiogram" and by respiration which sets the limit of the smallest twitch which can be recorded Ordinate Displacement of the tip of the needle in the tendon in μ Subject V. H, female, 17 years old (37° C)

iv) *Spontaneous activity, fasciculation* When a relaxed arm is kept in a forced position for an hour or more, the brachial biceps muscle often fasciculates In 2 subjects, in one of them during sleep, 50—60 twitches of the same fasciculating muscle fibres were averaged with the procedures described above (iii)

4 *Absolute values of force*

The absolute force of fibre bundles in the brachial biceps or the anterior tibial muscle was measured at the wrist or at the first metatarsus A steel rod, 8 mm in diameter, was pressed rigidly onto the inferior end of the radius or on the base of the first metatarsus The force was measured by a semiconductor strain gauge cemented on the rod and was recorded simultaneously with the displacement of the needle in the tendon The force of the adductor muscles of the thumb was measured by a condenser myograph connected to the thumb (Slomić, Rosenfalck and Buchthal 1968)

5 *Evoked muscle action potentials*

Muscle action potentials recorded with a concentric electrode ensured that the stimulus was activating new fibre bundles when the stimulating electrode was moved in steps across the end plate zone or when the stimulus was increased slightly The recording electrode was maintained at the same place and new activated fibre bundles gave a change in shape and size of the muscle action potentials

In some instances we wished to judge, from the amplitude of the summed action potential the number of muscle fibres activated To this purpose we inserted a steel wire, 80 μ in diameter through the cross section of the muscle and recorded between it and a needle electrode placed

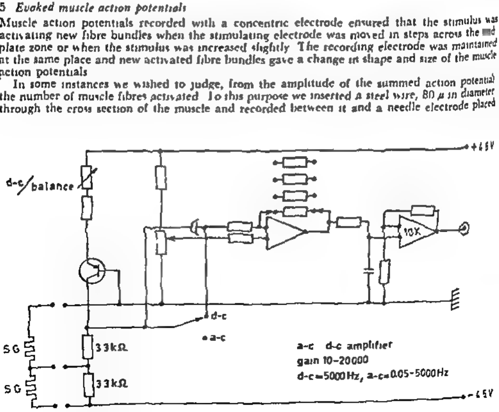


Fig 3 Bridge and amplifying circuit used with the semiconductor strain gauges

subcutaneously at a transverse distance of 6—10 cm By leading-off between the needle and another remote electrode it was ascertained that the pick-up by the needle electrode was negligible The impedance of the wire was reduced to about 500 Ω (35—350 Hz) by passing an a—c current of 50 mA through it for 30 sec (Buchthal and Rosenfalck 1966)

6 Recording and measurement

The output from the point between the two semiconductors (—2 25 V) was compensated to ... The amplifier ... out gave 1 V ... —c coupled,

The output of the mechano-electrical transducer in the tendon was displayed on one beam of a storage oscilloscope (time base 200 or 500 msec, Tektronix 564) and photographed with a Polaroid camera The muscle-action potentials, the output from the transducer on the wrist, foot or thumb, or the stimulating current was displayed on the second beam The traces were also displayed on a 3 channel electromyograph and photographed on paper (DISA 14A30)

The contraction time was measured as the time from the initial deviation from the base line to the crest of the force The amplitude of the muscle action potential was measured peak to-peak

7 Subjects and procedure

The experiments were performed on 25 subjects, 21 males and 4 females, without signs or symptoms of neuromuscular disease 21 were 16—20 years old and 4 were 45 to 63 years old

of a change in temperature the muscle was cooled by placing ice bags around it When an intramuscular temperature of 22° C was reached the ice bags were removed and the contrac-

Results

Comparison of the twitches recorded from the tendon and by conventional methods

1) Twitches of the adductor pollicis muscle

In 6 subjects twitches were evoked by stimuli to the ulnar nerve and the contraction time was recorded simultaneously with a transducer on the thumb and in the tendon of the adductor pollicis muscle near the head of metacarpus I

a *Time course* The time to peak was the same by the two procedures (Table I) and it did not vary systematically with the force In a given twitch the deviation was at most 2 msec Also the time to 75 % relaxation was in the same range with the two procedures (110 msec to 75 % relaxation)

b *Force* The transducer in the tendon did not give a measure of the absolute force as did the transducer on the thumb The tip of the needle was displaced the more the further it was from the point of insertion of the tendon At a given posi-

TABLE I Contraction times in twitches of the adductor pollicis muscle evoked by stimuli to the ulnar nerve (35° C, 6 subjects)

Twitch force	n	Time to peak contraction in msec measured from the tendon msec	S D msec	thumb msec	S D msec
Maximal	6	49 5	3	51 0	3
10—30 % of max	28	51 5	8	51 0	7 5

tion of the needle in the tendon the relative increase in amplitude obtained with an increase in stimulus strength was the same by both methods (Fig 4)

ii) Twitches of the brachial biceps muscle

In 9 subjects twitches were elicited by stimuli in the end plate zone and were recorded from the tendon and from a semiconductor strain gauge at the wrist (Slomic et al 1968)

a *Time course* The time to peak was slightly longer when recorded at the wrist than in the tendon because of the greater inertia (inset Fig 5)

b *Force* The twitch amplitude recorded in the tendon increased linearly with the force at the wrist up to 10—20 % of the maximum force of a twitch Above 20 % the amplitude recorded from the tendon increased less than the force recorded at the wrist because of lateral displacement of the tendon Fig 5 illustrates this deviation from linearity and shows that the amplitude of the summed action potential increased linearly with the force

The smallest force which could be measured with the transducer in the tendon was 0 5—1 % of the maximum twitch force (Fig 6 b c) and the force at the wrist was then not measurable Since the brachial biceps contains about 700—800 motor units (Buchthal 1961) and since the cross sectional area of the two heads is about the same then the smallest force corresponded to the average of 1 or 2 motor units The smallest force was reproducible when the twitch could be placed between oscillations of the ballistocardiogram

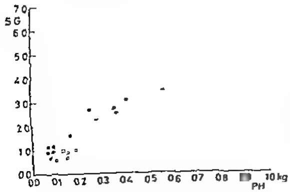


Fig 4 Twitch amplitudes recorded with the transducer in the tendon (SG) of the adductor pollicis muscle as a function of the force recorded simultaneously by a transducer PH connected to the thumb Stimuli of different strength to the ulnar nerve 3 subjects the intramuscular temperature was 35° C

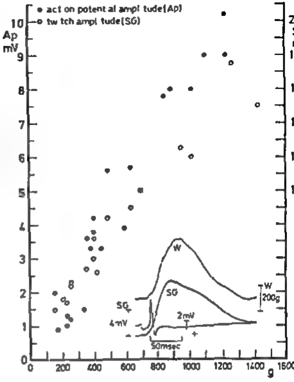


Fig 5 Twitch amplitude in m biceps brach cap long recorded with the transducer in the tendon (SG) and action potential amplitude (Ap) recorded with a wire electrode across the long head as a function of the force measured at the wrist (W) and calculated to the point of insertion of the tendon

Inset Twitch recorded simultaneously by the transducer in the tendon (SG) and the transducer on the wrist (W) and the evoked action potential

The maximal force of the twitch in the long head of the muscle was 3 4 kg the short head of the muscle did not contribute to the force as evidenced by the absence of an action potential in this part of the muscle The intramuscular temperature was 37° C

The force elicited by stimulation and by voluntary effort

The greatest twitch force evoked by a supramaximal stimulus to the end plate zone of the long head of the brachial biceps was 6 kg calculated to the insertion of the distal tendon and to 90° flexion at the elbow The maximum tetanic force was 35 kg and the twitch tetanus ratio was 0 17 The isometric force of the flexors during full voluntary effort in the same untrained subjects was 130 kg

The spectrum of contraction times

Weak stimuli applied to the end plate zone activated small bundles of fibres each with its contraction time (Fig 6) When the stimulating electrode was moved (a) or when the stimulus was increased slightly (b) a new fibre bundle could be activated

Repeated recording of the contraction times in the same muscle of the same subject on different days gave average values varying by 3—6 % (10—30 different fibre bundles), *i e* within the range to be expected from the standard deviation of the histogram determined in the single experiment The variation from subject to subject was about 10 % Contraction times and the range of the spectra were the same in males and females (m biceps br cap long males 51 4 ± 1 1 msec, n = 139, females 56 2 ± 2 1 msec n = 56) and independent of the subject's age (16—63 years) There

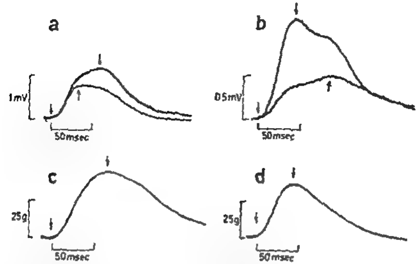


Fig. 8. Twitches of fibre bundles with different contraction times evoked by near threshold stimuli in the long head of the brachial biceps muscle (single sweep recordings, 37° C).
a: same stimulus strength applied in two points of the end plate zone (subject H. J., female, 18 years old).
b: stimuli of different strengths applied to the same point in the end plate zone (subject P. N., male, 19 years old).
c and *d*: twitches evoked by stimuli applied to two points of the end plate zone (subject O. C., male, 23 years old).
Ordinates: *a* and *b*: output of the strain gauge in mV, peak force 1—2 % of maximum twitch force; *c* and *d*: force in g, calculated to the insertion of the tendon at 90° flexion of the elbow, less than 1 % of maximum twitch force.

was no relation with the depth of the stimulating electrodes nor with the strength of the stimuli. Nor did the state of training alter the average and the range of contraction times. In a weight lifter, whose force was more than twice that of untrained subjects, the spectra of contraction times in the brachial biceps and triceps muscles were within the limits of untrained subjects. This is consistent with findings in adult rat muscles that training affects the different types of fibres to the same degree (Mai, Ito and Kikuchi 1967).

The spectrum of contraction times in the muscles examined

The contraction times were different in the muscles examined (Fig. 7 and 8). The distribution was normal, the skewness and the kurtosis being within the normal range. In the brachial biceps muscle the contraction times covered a wide range, whereas they were mainly fast in the brachial triceps and in the platysma. Contraction times exceeding 60 msec occurred in a third of the fibre bundles in the brachial biceps muscle, in 2 % of the bundles in the lateral head of the triceps and in none in the platysma (Fig. 7). In the muscles of the leg half of the contraction times in the anterior tibial muscle were slower than 60 msec and most of the fibres in the gastrocnemius and soleus muscles were slower than 60 msec (Fig. 8). The relative scatter

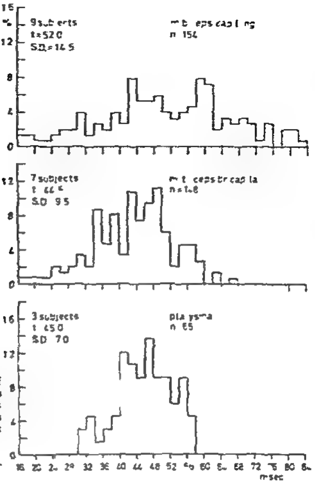


Fig 7 Average distribution of contraction times in small bundles of m. biceps brach., m. triceps brach. and platysma. n denotes the number of fibre bundles t the time to crest in msec and S.D the standard deviation in msec Intramuscular temperature 36—37° C.

(S.D.) of contraction times was greater in the brachial biceps and triceps (20—25 %) than in the muscles of the leg and in the platysma (10—12 %).

Contraction times and histochemical findings

The distribution of contraction times was related to the incidence of fibres of different type as identified histochemically by the amount of mitochondria. Slow contraction times corresponded to the incidence of fibres rich in mitochondria (type C) and fast contraction times to fibres with a medium amount of mitochondria (type B) and to fibres poor in mitochondria (type A) (Fig 9).

One of us (Schmalbruch 1970) has classified and counted 700 fibres from 2 regions of each of six human muscles (Table II). In the muscles of the triceps about 90 % of the fibres were rich in mitochondria (type C). 90—95 % of traction times of small bundles of muscle fibres in these muscles had cr

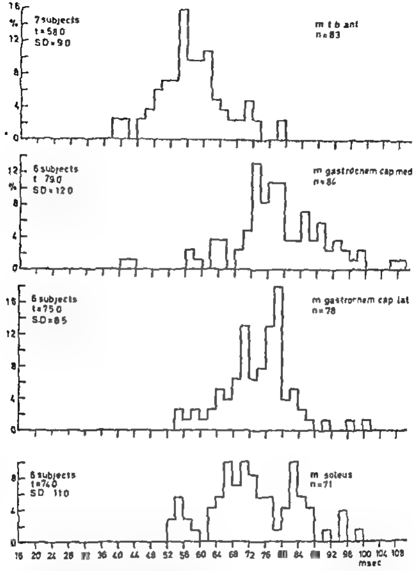


Fig 8 Average distribution of contraction times in small bundles of m tibialis ant and m triceps surae n denotes the number of fibre bundles t the time to crest in msec and SD the standard deviation in msec Intramuscular temperature 36—37° C

over 60 msec (Fig 8) In two anterior tibial muscles about half of the fibres were of type C and half of the contraction times were longer than 60 msec (Fig 8) Four teen human brachial triceps muscles were examined in the same way (Table II) Nearly all the fibres were poor in mitochondria (type A) or of intermediate type (type B) Nearly all the contraction times were shorter than 60 msec (Fig 7) In the brachial biceps muscles a quarter of the fibres were rich in mitochondria (type C), about 30 % of the contraction times were longer than 60 msec

o T T h d f a h h w w f h m sta ned weakly (type A)

usely sta ned fibres (type C)
urs after death sta ned by

5

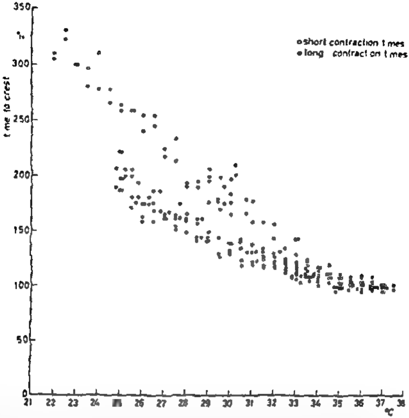
The shape of the twitch

The shape of the twitch depended on the contraction time and on the muscle exam ned In the muscles with both fast and slow contraction times the brachial biceps d the anterior tibial one third of the twitches had an initial fast and a later slow se in force (Fig 6 a) In muscles with mainly fast contraction times (lateral head of he triceps and platysma) and those with mainly slow contraction times (muscles of the calf) two distinct phases in the rising phase occurred in at most 10 % of the

TABLE II F bre types* per cent (Schmalbr ch 1970

Mus le	Number of muscles	Poor in m tochond A	S D	Intermed B	S D	R ch in m tochond C	S D
ceps b cap long	14	43	12 5	32	9 5	25	10
ceps br cap lat	14	50	17 5	48	17	[illegible]	1
b al s ant	2	19		35		46	
s rocnem us cap lat	6	10	12	8	13	[illegible]	10
med	5	7	[illegible]	9	13	84	10
oleus	6	4	7	7	11	89	10

*D fferen a ed by Sudan black B Gau h er & Padykula 1966
00 fibres f om d fferent reg ons of each muscle



ig 10 Contraction times as a function of temperature in 2 fibre bundles with short contraction times (44 and 46 msec at 37° C m triceps cap lat) and 3 fibre bundles with long contraction times (82 80 58 msec at 37° C m gastrocnemius cap med) Ordinate time to crest expressed in % of the time at 37° C Abscissa Intramuscular temperature

twitches A hump during relaxation (Gordon and Phillips 1953) was recorded in one fourth of the twitches recorded in the biceps triceps and platysma and in only one tenth of the twitches in the calf muscles

Pronounced inflexions in the time course as they would result from a mixture of fibres with fast and very slow contraction times (Biscoe and Taylor 1967), were recorded rarely and then only in the brachial biceps and the anterior tibial muscle

Effect of temperature

Between 22 and 32° C the contraction time of fast fibre bundles decreased by 10 % per °C ($Q_{10}=2\ 5$) The time to peak force of slow fibre bundles decreased by 7 % per °C ($Q_{10}=2\ 0$) (Fig 10) Above 32° C the decrease was 5 % per °C in fast and 4 % per °C in slow fibre bundles ($Q_{10}=1\ 7$ and 1 5) The peak force of the twitches varied randomly by 10 % throughout the 3 hrs of an experiment

Since the change in contraction time with temperature was larger in fast than in slow fibre bundles the spectrum of contraction times was narrower at 22—24° C (S D 10 %) than at 36—38 C (S D 22 % Fig 11)

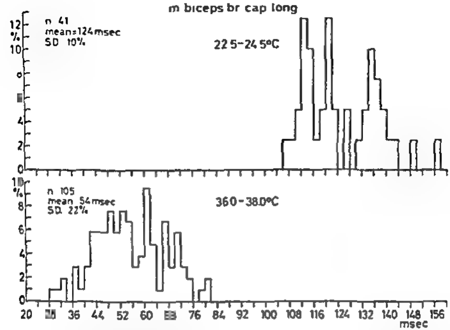


Fig 11 Spectra of contraction times at low (above) and at normal temperature (below) (2 subjects) n denotes the number of bundles Note that the relative standard deviation was halved in the spectrum at low temperature

Effect of circulatory arrest (Fig 12)

Hypoxia caused by circulatory arrest for 20—45 min eliminated the slower contraction times in each muscle and shifted the peak of the spectrum to fast contraction times Thus in the brachial biceps which before hypoxia had 30 % contraction times above 60 msec, there were virtually none during hypoxia and contraction times below 46 msec, nearly absent before hypoxia accounted for a third of contraction times ($p<0\,001$) Similarly nearly all contraction times in the gastrocnemius were above 70 msec and up to 104 msec during hypoxia half were slower than 70 msec and none was longer than 86 msec ($p<0\,001$) In the brachial triceps which normally has fast contraction times the slowest times were eliminated during hypoxia and the peak of the spectrum was shifted to the fast times but no fibre bundles contracted faster than normal for the triceps

Twitches of single motor units and of fasciculations

Three subjects could activate single motor units in the brachial biceps muscle voluntarily at 3 to ▮ per sec The contraction times of these firstly activated motor units were 40—65 msec (5 motor units 36 5° C Fig 13)

In 2 subjects in one of them during sleep we averaged 50—60 fasciculations accompanied by the same action potential and therefore originating from the same bundle of muscle fibres The force of these twitches was about th

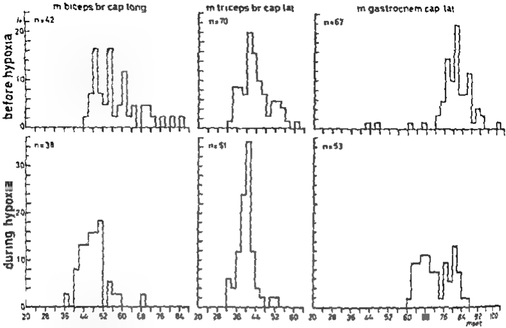


Fig 12 Spectra of contraction times before and during hypoxia obtained by interrupting the circulation for 20—40 min
Each histogram was averaged from 2 normal subjects 18 years old

tor units activated firstly during weak effort The contraction times of fasciculating idles were similar to those of the single motor units (35—70 msec 36 5° C, 8 asciculating bundles Fig 13)

Discussion

Contraction time in whole and in excised human muscle

Contraction times have been measured indirectly in intact human muscles (*Buller et al* 1959, McComas and Thomas 1968) and directly in excised samples of human muscles (Brust and Cosla 1967, Eberstein and Goodgold 1968) and it has been established that different muscles have different contraction times ranging from about 40—120 msec Only few of these contraction times were measured from those muscles we have studied They fall in about the middle of the spectrum we obtained by recording from 20—30 small bundles of fibres in each of 7 muscles of normal subjects The division between 'fast' and "slow" muscles was about 60 msec in the muscles we examined The fast muscles, the platysma and the brachial triceps, had contraction times shorter than 60 msec, the slow muscles, gastrocnemius and soleus had times longer than 60 msec

In the excised samples of muscles the mean contraction time of 'fast' muscles was 64 msec and of 'slow' muscles 120 msec From the force they measured it appears that Eberstein and Goodgold's (1968) samples were hypodynamic (maximum tetanic

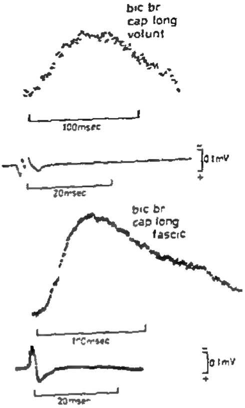


Fig 11 Above Average of 200 twitches (3—6/sec) in a single motor unit of the brachial biceps muscle during weak voluntary effort
Below Average of 64 identical fasciculations
Normal subjects, intramuscular temperature 36—37° C. Note the different time base of the action potentials and the twitches.

force 0.4 kg/cm² as compared to 6 kg/cm² in normal muscle) This as well as the stiffness of an unknown amount of non-responsive muscle tissue in series with the contracting fibres may account for the longer contraction times of excised muscle

Contraction times and histochemical findings

There is abundant evidence that mammalian slow muscles differ from fast muscles in electronmicroscopic and histochemical appearance These features have been related to contraction times in animals but hitherto not in man In our normal subjects there was a clear general relation between slow contraction times and type C fibres on the one hand, and fast contraction times and A and B fibres on the other

Experiments with hypoxia confirmed the relation of type C fibres and long contraction times Since the C fibres are rich in oxidative enzymes, they should be more susceptible to hypoxia than A fibres whose metabolism is glycogenolytic In fact arrest of the circulation to the extremity cut out the longest contraction times and shifted the distribution of contraction times to shorter values, some shorter than appeared in those muscles before hypoxia (Fig 12) That the slow bundles of fibres responded with a twitch at all during hypoxia is consonant with the observation that

individual fibres of each type show distinct heterogeneity for oxidative and glycogenolytic enzymes (Stein and Padykula 1962, Romanul 1964).

The small though significant decrease in contraction time in the brachial triceps muscle could be due to loss of the intermediate fibres (type B). They contain oxidative enzymes and are fast but presumably not as fast as fibres of type A.

The different metabolism of the fast and slow fibres in human muscle is reflected in the different temperature dependence. Fast fibres had a higher temperature coefficient ($Q_{10}=2.5$) than the slow fibres ($Q_{10}=2.0$) in the range 22 to 32° C, 1.7 and 1.5 respectively in the range 32—38° C. This Q_{10} is close to the 1.55 found by Gordon and Phillips (1953) in a relatively fast muscle in the cat. In the rat the opposite has been reported, a higher temperature coefficient for slow than for fast fibres (Close and Hoh 1968), whereas in the cat the temperature dependence was the same in fast and in slow muscle (Buller, Ranatunga and Smith 1968).

Contraction times and histochemical findings in cat and man

In man, then, a relation between type C fibres and long contraction times is clear and type B fibres seem to resemble type A fibres in giving short contraction times. Similarly in the cat the contraction times of single motor units were predominantly fast in a muscle, the medial head of the gastrocnemius (Wuerker, McPhedran and Henneman 1965), which contains about 50 % type A fibres and 25 % type B fibres (Henneman and Olson 1965), a qualitative resemblance to the relations in human muscle described above.

A conflicting report describes the slow red soleus muscle of the cat as consisting entirely of B fibres (Henneman and Olson 1965), though the contraction times were even somewhat longer than in man (McPhedran, Wuerker and Henneman 1965).

The range of contraction times, real or composite?

In the cat each motor unit is composed of fibres uniform in type (Doyle and Mayer 1969) confirming findings in rat muscle (Brandstater and Lambert 1968, Edström and Kugelberg 1968). Each motor unit has its characteristic contraction time (Henneman and Olson 1965). Since we stimulated small bundles of fibres which may or may not belong to the same motor unit we have surely in some instances recorded a composite twitch of fast mixed with slow fibres. In a third of the fibre bundles in heterogeneous muscles, the brachial biceps and anterior tibial muscles, the rising phase of the twitch was composite or there was a hump during relaxation, indicating that fibres with different contraction times were in the bundle activated. Such composite contractions were rare in other muscles and we believe that most contractions were produced by fairly homogeneous fibres and that there is a real range of contraction times in the muscles we studied. Three main reasons for our belief are given below. i) The single motor units we recorded from were those activated voluntarily in the biceps during weak voluntary effort. They had contraction times of 60—65 msec, in the middle of the spectrum of that muscle, so there is at least one real contraction time between the fast and the slow. ii) The soleus muscle of cat contains a

uniform population of fibres (Henneman and Olson 1965) The single motor units had slow contraction times over the range from 50 to 150 msec (McPhedran Wuerker and Henneman 1965) thus certainly not a composite of fast and slow The peak we found in human soleus with an almost uniform population of C fibres (Table II) was slightly faster and the range was less than in cat m) Pronounced inflections on the course of the twitch were seen only rarely If the twitches were the result of a mixture of only two contraction times as in the intercostal muscles of the cat (Andersen and Sears 1964) this would appear in the complex slope of tension development or relaxation (Biscoe and Taylor 1967)

The force of the evoked twitches of voluntary effort and of fasciculations

The maximum tetanic force evoked by electrical stimuli was 35 kg that of the flexors during full voluntary effort was 130 kg Since the long head of the brachial biceps contributes 25 % to the force exerted by the brachialis and the biceps muscles (Fick 1911) the force was the same in electrically and in voluntarily evoked contraction as shown by Merton (1954) in the adductor pollicis muscle The force of 130 kg during flexion gives a force per unit area of 6—7 kg/cm² assuming a cross sectional area of the flexors of the upper area of 20 cm² (determined by ultrasound Ikai and Fukunaga 1968) This is a force within the range found by Ikai and Fukunaga (1968) In fact the maximum isometric force per unit area of the brachialis and brachial biceps muscles was slightly less 5—6 kg/cm² when corrected for the 20 % which the brachioradial muscle contributes to flexion (Fick 1911)

The force of fasciculations was three times that of the single motor unit This can either mean that fasciculating bundles contain fibres from several motor units or that fasciculations represent larger motor units than those initially activated during weak voluntary effort

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References

ABRAHAMS M ... and ... *J Med ... Engng* 1967 5 433—449

B[illegible]

BOTELHO ... ischemia in intact hum[illegible]

BRAND[illegible] ... ial arrangement of mus[illegible] ... *Il Amer Ass Elec* ... from ... ral 1968 Chic Ill 196[illegible]

BRUST M and H W COSTA Contractility of isolated human skeletal muscle *Arch phys Med* 1967 *48* 543—555

BUCHTHAL F The general concept of the motor unit chapter 1 in Neuromuscular [illegible] ed by R. D Adams L M Eaton and G M Shy *Res Publ Ass nerv ment Dis* 3—30

BUCHTHAL, F and A ROSENFALCK, Evoked action potentials and conduction velocity in human

B

B

muscles in man ... *Nature* (Lond) 1959 183 1316—1317

BULLER, A. J., K. W RANATUNGA and J SMITH, Influence of temperature on the isometric myograms of cross innervated mammalian fast twitch and slow twitch skeletal muscles. *Nature* (Lond) 1968 *218* 877—878

CLOSE, R. AND J F Y HOH Influence of temperature on isometric contractions of rat skeletal muscle *Nature* (Lond) 1968 *217*, 1179—1180

DESMEDT, J E Methodes d'étude de la fonction neuromusculaire chez l homme *Acta neurol belg* 1958 *58* 977—1017

DOYLE A M and R. F MAYER, Studies of the motor unit in the cat *Bull Sch Med Maryland* 1969 *54* 11—17

EBERSTEIN A. and J GOODGOLD, Slow and fast twitch fibers in human skeletal muscle *Amer J Physiol* 1968 *215* 535—541

EDSTRÖM, L. and E KUGELBERG, Histochemical composition, distribution of fibers and fatiguability of single motor units. *J Neurol Neurosurg Psychiat* 1968 *31* 424—433

FICK R., Handbuch der Anatomie und Mechanik der Gelenke Spezielle Gelenk- und Muskelmechanik in v Bardeleben, K *Handbuch der Anatomie des Menschen* Bd. 2, I Abtlg 3 Teil Fischer, Jena, 1911 pp 688

GAUTHIER, G F and H. A. PADYKULA, Cytological studies of fiber types in skeletal muscle *J Cell Biol.* 1966 *28* 333—354

GORDON, G and C G PHILLIPS, Slow and rapid components in a flexor muscle *Quart J exp Physiol* 1953 *38* 35—45

GUTH, L, Trophic influences of nerve on muscle *Physiol Rev* 1968 *48* 645—687

HENNEMAN, E and C B OLSON, Relations between structure and function in the design of skeletal muscles *J Neurophysiol* 1965 *28* 581—598

IKAI M and T FUKUNAGA Calculation of muscle strength per unit cross-sectional area of human muscle ...

LAMBERT, E. H ...
sionally associ ...
Myasthenia gr ...

MANN M K I ...
of training up ...
153—165

McCOMAS A J and H C THOMAS Fast and slow twitch muscles in man *J Neurol Sci* 1968 *7* 301—307

McPHEDRAN A M R B WUERKER and E HENNEMAN, Properties of motor units in a homogeneous red muscle (soleus) of the cat *J Neurophysiol* 1965 *28* 71—84

MERTON P A. Voluntary strength and fatigue *J Physiol (Lond) 1954 123* 553—564

NISSEN-PETERSEN H C GUILD and F BUCHTHAL A delay line to record random action potentials *Electroenceph clin Neurophysiol* 1969 *26* 100—106

ROMANUL F C A Enzymes in muscle I Histochemical studies of enzymes in individual muscle fibers *Arch Neurol* (Chic) 1964 *11* 355—368

SCHMALBRUCH H Die quergestreiften Muskelfasern des Menschen *Ergebn Anat EntwickL-Gesch* 1970 *43/1* In press

SLOMIĆ, A A ROSENFALCK and F BUCHTHAL, Electrical and mechanical responses of normal and myasthenic muscle with particular reference to the staircase phenomenon *Brain Res* 1968 *10* No 1 spec iss pp 1—78

STEIN, J M and H A PADYKULA, Histochemical classification of individual skeletal muscle fibres of the rat *Amer J Anat* 1962 *110* 103—124

WÖHLISCH E R. DU MESNIL DE ROCHEMONT and H GERSCHLER Untersuchungen über die elastischen Eigenschaften tierischer Gewebe I Elastizitatsmodul, Zerreissfestigkeit Arbeitsvermögen und elastische Vollkommenheit *Z Biol* 1926 *85* 325—341

WUERKER, R. B A M McPHEDRAN and E HENNEMAN Properties of motor units in a heterogeneous pale muscle (M gastrocnemius) of the cat *J Neurophysiol* 1965 *28* 85—99

Acta physiol. scand. 1970. 79. 453—461

From the Zoophysiological Laboratory A, University of Copenhagen, Denmark

Sodium Transport and D.C. Resistance in the Isolated Toad Skin in Relation to Shedding of the Stratum Corneum

By

ERIK HVIID LARSEN

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Abstract

HVIID LARSEN, E. *Sodium transport and D.C. resistance in the isolated toad skin in relation to shedding of the* s t r a t u m   c o r n e u m. Acta physiol. scand. 1970. 79. 453—461.

It has been assumed that the main resistance to sodium ion movements is located in the superficial layer of the amphibian skin. It is therefore of interest to follow transport function during shedding of the *stratum corneum*. Shedding of the *stratum corneum* in the isolated toad skin (*Bufo bufo*) can be induced by aldosterone treatment of skin from hypophysectomized toads. It is shown that slough formation is followed by an almost three fold increase in short-circuit current. This increase is accounted for by an increased rate of active sodium transport and is preceded by an increase in passive sodium permeability and a decrease in D.C. resistance. The results are discussed in relation to the functional organization of the skin, and to mounting *in vitro*.

The isolated amphibian skin mounted as a membrane between two chambers containing Ringer's solution carries out an active transport of sodium from the solution bathing the outside to that bathing the inside of the skin, and secondary to this a passive chloride transport takes place in the same direction. In the two-membrane hypothesis put forward by Koefoed-Johnsen and Ussing (1958) this asymmetric function was assumed to be located to the *stratum germinativum* cell, the apical and basal unit membranes of which were considered as being functionally different. It has been verified in later studies that the skin consists of two functionally different membranes in series (MacRobbie and Ussing 1961, Hoshiko 1961, Ussing 1965), but it is now evident that to regard the skin as consisting of only one functional cell layer is an oversimplification (Whittembury 1964, Ussing and Windhager 1964, Cereijido and Curran 1965, Biber, Chez and Curran 1966, Farquhar and Palade 1966, Voute and Ussing 1968). According to the revised two-membrane hypothesis (Ussing and Windhager 1964, Farquhar and Palade 1966) the overall transport is brought by an interplay between several cell layers of the epithelium. Accordingly

ward facing membrane' is located just beneath the cornified layer, and the 'inward facing membrane" is identical with the membranes limiting the extracellular spaces throughout the epithelium Thus the asymmetric nature of the epithelium must be assumed to be developed by a process of differentiation as the cells move outward and is not the result of an inherent functional asymmetry of single germinativum cells Looking at the functional organization of the skin from this point of view, we may ask whether it is possible to correlate processes of differentiation during the moulting cycle with simultaneous changes in functional characteristics such as ion selectivity and transport rate

The toad moults about every ten days During this process the outermost cornified cell layer loses contact with the deeper part of the epithelium forming a continuous slough The slough is shed and swallowed by the animal In the present study transport function is studied in the isolated toad skin during shedding of the *stratum corneum* A preliminary report on this work was given at the Fifth Conference of European Comparative Endocrinologists held in Utrecht in 1969 (Hvid Larsen 1969)

Materials and Methods

The rate of active charge transport was measured by the short-circuit technique developed by Ussing and Zerahn (1951 In the first experiments, short-circuiting was done manually and the skin was mounted in a conical chamber with an exposed area of 7 1 cm^2 (Koefoed Johnsen Ussing and Zerahn 1952) In later experiments an automatic voltage clamping apparatus was used (Bengtson and Hvid Larsen to be published) This equipment provides for continuous recordings of clamping current and voltage, and for intermittent recording of spontaneous potential The skin was mounted in a conical double chamber and for each half of the skin the exposed area was π cm^2 The electrodes (Ag/AgCl half cells) for passing a current through the skin were placed directly in the solutions bathing the skin The agar Ringer bridges for measuring the potential were placed in close contact with the skin surfaces to eliminate gross errors in short-circuiting due to the voltage difference in the Ringer's solution between the tips of the bridges and the skin surfaces In addition in the experiments where tracer flux was measured the skin was clamped at a reversed voltage (0 to 1 5 mV depending on current) which exactly balanced the voltage difference between the tips of the bridges Aeration and circulation of the bathing solutions were maintained as described by Koefoed Johnsen Ussing and Zerahn (1952

The *D C resistance* of the skin was determined as the ratio of the open circuit potential to the short-circuit current

It is assumed that the *sodium permeability* of the skin can be expressed as the passive sodium ou [illegible]
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ou [illegible]
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Induction of slough formation Jørgensen and Larsen (1964) have shown that in the hypophysectomized toad a complete moult can be induced by injecting a corticosteroid 24 hrs after the operation The moult then takes place 6—7 hrs after injection of the hormone In the present study the hormone is added to the solution bathing the inside of the isolated skin

Twenty to twenty four hours after removal of the pars distalis of the hypophysis from the toad (*Bufo bufo* (L) performe [illegible] the abdominal skin was removed and mounted [illegible] equilibration *aldosterone* (Aldocorten Ciba [illegible] ncentration of 2 7 × 10 [illegible] moles/l The slough formation was [illegible] from the same

TABLE I Net Na flux and short circuit current (scc) before and after slough formation in toad skin

	before (n = 15)	after (n = 30)
	μeq -cm² hr	
Na influx	3 22±0 27*	4 64±0 21
Na outflux	0 40±0 10	0 97±0 12
net Na flux	2 82±0 27	3 69±0 24
scc	2 87±0 23	3 63±0 22

* mean ± S E M

toad by prodding the *stratum corneum* with a watch maker s forceps at intervals of about half an hour under a Zeiss stereo-microscope using 16× magnification
Experimental groups G
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Fig 1 *Group B* In 8 d
tinuously and sodium o
treated membranes) as well as in the control skin halves An example of this type of experiment is represented in Fig 4 *Group C* The net sodium flux was determined in 6 expts on automatically and continuously short-circuited membranes The results are represented in Table I

Results

1 *The Effect of Slough Formation on the Short circuit Current and the D C Resistance*

Fig 1 shows the result of a single experiment Enhancement of the current does not occur during the first five to six hours after hormone treatment but as the *stratum corneum* is shed the current increases This increase in current is preceded by a decrease in resistance In Fig 2 completion of slough formation has been denoted zero time and current as well as resistance are expressed relative to the values at this time It appears from the figure that shedding of the *stratum corneum* is followed by an almost three-fold increase in current A comparison of the absolute resistance values before and after slough formation reveals that the decrease in resistance amounts to almost 60 % of the total skin resistance indicating that the resistance of the *stratum corneum* dominates the overall skin resistance

In the present series of 10 expts , the skin resistance before formation of a slough varied from 3 3 to 2 5 kΩ cm which illustrates the great variation of this parameter In skins showing the highest and lowest initial values the drop in resistance following shedding was 5 3 and 0 3 kΩ cm respectively which seems to indicate that the variability of the skin resistance is due largely to that of the *stratum corneum* resistance

In Fig 3 the resistance drop (ΔR) following shedding of *stratum corneum* is plotted against the resistance before shedding The correlation between ΔR and total skin resistance is evident (r = 0 970) The time lapse between the last moulting *in vivo* and the isolation of the skin was not determined and therefore it remains unc

ward facing membrane" is located just beneath the cornified layer, and the inward facing membrane" is identical with the membranes limiting the extracellular spaces throughout the epithelium. Thus the asymmetric nature of the epithelium must be assumed to be developed by a process of differentiation as the cells move outward and is not the result of an inherent functional asymmetry of single germinativum cells. Looking at the functional organization of the skin from this point of view, we may ask whether it is possible to correlate processes of differentiation during the moulting cycle with simultaneous changes in functional characteristics such as ion selectivity and transport rate.

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The *D.C. resistance* of the skin was determined as the ratio of the open circuit potential to the short circuit current.

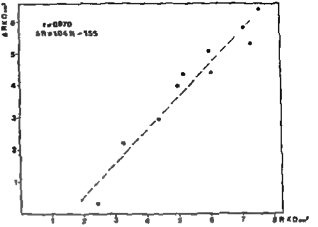
It is assumed that the *sodium permeability* of the skin can be expressed as the passive sodium outflux in the short circuited skin. [illegible] seems to be fulfilled in the isolated [illegible] added to the inside solution and [illegible] and inside solutions were drawn eve[illegible] System. The *net sodium flux* was measured as the difference between ^{22}Na influx and ^{24}Na outflux according to the method described by Koefoed Johnsen, Ussing and Zerahn (1952).

Ringer's solution of the following composition was used: 113.5 meq Na⁺/l, 1.9 meq K⁺/l, 1.8 meq Ca⁺⁺/l, 114.8 meq Cl⁻/l and 2.4 meq HCO₃⁻/l, pH = 8.2 when aerated with atmospheric air.

Induction of slough formation. Jørgensen and Larsen (1964) have shown that in the hypophysectomized toad a complete moult can be induced by injecting a corticosteroid 24 hrs after the operation. The moult then takes place 6—7 hrs after injection of the hormone. In the present study the hormone is added to the solution bathing the inside of the isolated skin.

Twenty to twenty four hours after removal of the pars distalis of the hypophysis from the toad (*Bufo bufo* (L.)) performed according to Jørgensen and Larsen (1961), the abdominal skin was removed and mounted in the flux chamber. After one to two hours of equilibration *aldosterone* (Aldocorten Ciba) was added to the inside solution to a final concentration of 2.7×10^{-7} moles/l. The slough formation was followed in a piece of belly skin from the same

Fig 3 Resistance drop ΔR following shedding of the *stratum corneum* as a function of total skin resistance R before shedding
Correlation coefficient and equation for regression line at top
$SD_{slope} = 0.52$
$SD_{intercept} = 0.09$ kΩ cm²



3 *The Effect of Slough Formation on the Sodium Permeability*

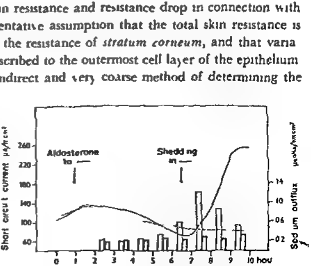
It appears from Table I that the passive sodium outflux likewise increases as slough formation is completed To investigate this phenomenon in detail, current and Na⁺-outflux were measured in a double chamber The result of a single experiment is depicted in Fig 4, and eight experiments of this type are shown in Fig 5, from which it is evident that the shedding of *stratum corneum* is preceded by a pronounced reversible increase in the sodium outflux Since these measurements were made on short circuited skins, we may conclude that the change in the sodium outflux is due to a reversible increase in sodium permeability

Discussion

1 *The Drop in D C Resistance*

The correlation between total skin resistance and resistance drop in connection with shedding (Fig 3) leads to the tentative assumption that the total skin resistance is determined to a large degree by the resistance of *stratum corneum*, and that variations in skin resistance must be ascribed to the outermost cell layer of the epithelium The results obtained with this indirect and very coarse method of determining the

Fig 4 Short-circuit current (curves) and sodium outflux (bars) in paired pieces of skin from a hypophysectomized toad (group B) Aldosterone was added to the experimental skin (full curve white bar) at 1 h and slough formation completed at the second arrow In the control skin (dashed curve, lined bars) the *stratum corneum* was intact (no slough formation)



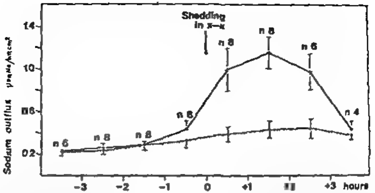


Fig 5 Sodium outflux in paired pieces of skin from eight hypophysectomized toads (group B) In each experiment aldosterone was added to one membrane (x—x), the other serving as control (o—o) The sodium outflux is depicted relative to the time of shedding (zero time) in the aldosterone treated membranes The *stratum corneum* was intact in the control membranes (no slough formation) Vertical bars indicate ± S E M

resistance profile of the outer region of the epithelium must be verified by direct electric measurements According to the literature, however, the resistance profile of the isolated skin is poorly known Hoshiko (1960 and 1961) and Cereijido and Curran (1965) determined the profile in skins of *Rana pipiens*, and found that 70—80 % of the resistance was measured between a microelectrode located in the intra cellular space of the epithelium and the outside solution As the localization of the microelectrode within the skin was not determined precisely, it is unknown how many llular layers were included in this outer part of the epithelium More valid support of the above assumption is given in the work of Lindemann and Thorns (1967) By inserting a microelectrode into the superficial cell layer of the isolated skin from *Rana esculenta*, the authors found that "the main resistive barrier appeared to be at the outermost surface of the epithelium" Moreover, they noticed that the resistance of the superficial layer varied from skin to skin which they ascribed to variable differentiation of this layer during the moulting cycle, as judged on the basis of its vital staining ability and the ease of tip penetration They claimed however that these superficial cells were never fully cornified and were "dying" cells Whether this observation is due to the cornified cells proper being lost in the preparations (as questioned by the authors themselves) or whether this mostly aquatic amphibian species has a poorly developed *stratum corneum*, remains obscure

Whittembury (1964) has shown that a resistance value for the *stratum corneum* of *Bufo marinus* could not be detected Since these determinations were made on skins with very low overall resistance ($1\,53 \pm 0\,42$ KΩ cm² (S D)) this finding is compatible with the above assumptions

2 *The Effect of Slough Formation on Sodium Permeability*

As the drop in resistance (Fig 1) as well as the increase in sodium permeability (Fig 4) preceded the increase in current it seems justified to assume that the drop in

resistance is due in part to an increased sodium conductivity An increase in permeability to other ions might also occur, as indicated by the fact that the sodium permeability decreased again after a few hours, whereas the resistance remained more or less constant and very low However, a further discussion or an interpretation of this relationship is hardly worth-while, because D C resistance and ion permeability were not studied in the same preparations

The pronounced increase in sodium permeability indicates that some structures within the *stratum corneum* have an important function in preventing sodium loss from the internal to the external environment According to the two-membrane hypothesis, the sodium diffusion barrier preventing sodium loss along the intracellular pathway is identical with the "inward facing membrane" i e the membranes lining the extracellular spaces throughout the epithelium In addition a barrier must suppress back diffusion of "transported sodium" from the extracellular spaces directly to the outside fluid According to Ussing and Windhager (1964) this barrier should be identical with the occlusion belts located in the apical part of the granulosa cells or the cornified cells The present findings are compatible with this view because sodium loss is markedly increased as *stratum corneum* is shed It is therefore tempting to conclude that it is *stratum corneum* or structures within this stratum (i e occlusion belts between *stratum corneum* cells or cement layer between the cornified cells and the granulosa cells) which is specialized for this function

3 *The Nature of the Increased Sodium Influx*

It is well established that aldosterone has a stimulating effect on the sodium transport in frog skin (Crabbé 1964) and toad bladder (Crabbe 1961) and that stimulation occurs within the first two hours after application of the hormone The increased sodium influx seen in connection with shedding of *stratum corneum* which occurred five to eight hours after addition of aldosterone could also be due to a direct hormonal effect on the sodium transport mechanism but if so we have to explain the long delay One possible explanation may be that the old *stratum corneum* limits the amounts of sodium that reaches the sodium pump As shedding eliminates this barrier more sodium will enter the skin and at this time the aldosterone-stimulating effect on the sodium transport mechanism shows as an increased active sodium influx This theory may be open to criticism because an increase in sodium permeability often precedes the increase in active sodium influx Moreover the increase in sodium permeability is totally reversible whereas this is not the case for the stimulation of the active sodium transport *The increased sodium permeability and the increased sodium influx should thus be more or less independent processes* According to Ussing and Windhager (1964) passively moving ions should mainly follow the extracellular pathway whereas the actively transported sodium exclusively follows the intracellular pathway If the resistance along the extracellular pathway changes without a simultaneous change in the resistance between the outer solution and the cellular compartment, we can explain why the time course of the passive permeability change is different from that of the change in the active sodium influx

4 *Comparisons with Moulting in Vivo*

Jørgensen (1949) has shown that anurans (*Rana esculenta, R temporaria* and *Bufo bufo*) leak sodium as well as chloride during moulting Also water permeability increases as observed by Jørgensen (1949) and by Ewer (1951) in *Bufo regularis* As Jørgensen moreover found an increased sodium influx during moulting, it indicates that the epithelium in these respects behaves in the same manner, both *in vivo* and *in vitro*

However, the normal moult *in vivo* differs in several aspects from the induced slough formation *in vitro* *In vivo* the *stratum corneum* begins to separate 1—2 days before the moult takes place, and just before shedding commences mucus is formed beneath the slough The origin of this mucus formation is not known In the present investigation the slough formation lasted 2—3 hours and mucus formation was never observed That the induced slough formation is a faster process than the spontaneous slough formation is also indicated by a longer duration of the increased permeability *in vivo*

The mechanism by which sodium influx is increased during moulting *in vivo* is obscure If the model developed for the *in vitro* preparation can be applied to the skin *in vivo*, an increased extracellular permeability could itself promote an increased transcellular active sodium influx by a so-called 'internal short circuiting' described by Voûte and Ussing (1968) Finally, part of the increased sodium influx could be due to a solvent drag effect, as the osmotic water uptake is very much increased during moulting

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References

Biber Th U L R A Chez and P F Curran Na$^+$ transport across frog skin at low external Na$^+$ concentration *J gen Physiol* 1966 *49* 1161—1176

Cereijido M and P F Curran Intracellular electrical potentials in frog skin *J gen Physiol* 1965 *48* 543—557

Crabbe, J Stimulation of active sodium transport by the isolated toad bladder with aldosterone *in vitro* *J clin Invest* 1961 *40* 2103—2110

Crabbe, J, Stimulation of active sodium transport across the isolated ventral skin of amphibia *Endocrinology* 1964 *75* 809—811

Ewer R. F, Water uptake and moulting in Bufo regularis Reus *J exp Biol* 1951 *28* 369—373

Farquhar M G and G E Palade Adenosine triphosphatase localization in amphibian epidermis *J cell Biol* 1966 *30* 359—379

Fed Pr 1960 19 177

al ac

e skin

9 171

—180

Jørgensen C Barker and L Olesen Larsen Moulting and its hormonal control in toads *Gen comp Endocr* 1961 *1* 145—153

JORGENSEN C BARKER and L OLESEN LARSEN Further observations on moulting and its hormonal control *Gen comp Endocr* 1964 *4* 389—400
current
physiol
scand 1958 42 298—300
LARSEN, E HVIID, *Active and passive sodium transport and chord resistance in the isolated short circuited toad skin during aldosterone induced slough formation* Fifth Conference of European Comparative Endocrinologists Utrecht 1969 (Abstract)
LINDEMAN B and U THORNS Fast potential spike of frog skin generated at the outer surface of the epithelium *Science* 1967 *3807* 1473—1477
MACROBBIE A C and H H USSING Osmotic behaviour of the epithelial cells of frog skin *Acta physiol scand* 1961 *53* 348—365
USSING H H Relationship between osmotic reactions and active sodium transport in the frog skin epithelium. *Acta physiol scand* 1965 *63* 141—155
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Acta physiol. scand. 1970. 79. 462—468

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Morphogenesis of Myoneural Junctions Induced Postnatally in the Tibialis Anterior Muscle of the Rat

By

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Abstract

JUNTUNEN J and H TERÄVÄINEN *Morphogenesis of myoneural junctions induced postnatally in the tibialis anterior muscle of the rat* Acta physiol scand 1970 79 462—468

The structure of myoneural junctions induced during nerve regeneration outside the zone of previously existing degenerating myoneural junctions was studied with the light microscope after histochemical demonstration of acetylcholinesterase (AChE) activity in the tibialis anterior muscle of the postnatal rat The escape of regenerating nerves from their original glial sheaths was produced by severe crushing of the sciatic nerve at different stages of development of the rat from newborn animals to adults New myoneural junctions were induced in the previously undifferentiated part of the muscle plasma membrane in animals of all age groups although not in every individual animal if the old myoneural junctions did not regenerate New myoneural junctions were observed in about 70 % of the rats operated on before the age 10 days and in less than 20 % in the older groups The older the animal the fewer new myoneural junctions were seen among the re innervated old ones and in the adult animals a new myoneural junction was only sporadically found

Myoneural junctions were first seen outside the zone of previously existing junctions 14—16 days after the operation Irrespective of the age of the animal at operation the postnatally induced myoneural junction was plate like in structure during the early stages of morphogenesis Postsynaptic infoldings appeared between the 16th and 25th days after the operation the infoldings had become deeper between the 25th and 35th days and the ramification of the AChE distribution typical of the mature myoneural junctions was observed between the 35th and 45th postoperative days

The appearance of locally increased AChE activity constitutes a histochemical sign of the formation of the primitive myoneural junction in the rat tibialis anterior muscle in utero at the 18th day of gestation Both histochemical (Teräväinen 1968a) and electron microscopic (Teräväinen 1968b Kelly and Zacks 1969) techniques demonstrate the appearance of the typical infoldings to the postsynaptic membrane about a week after the formation of the first plate like myoneural structure Thereafter the myoneural junction undergoes profound structural changes ultimately acquiring the co[illegible] ramified and segmented appearance of the mature neural synapse with [illegible] loped postsynaptic infoldings Similar morpho

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genetic events occur in human myoneural development but the time required is considerably longer (Juntunen and Teravainen 1969) A continuous neural influence seems to be essential for the development of the mature myoneural junction (Teravainen and Juntunen 1968)

As long ago as 1944, Gutman and Young using silver impregnation techniques observed that myoneural junctions possessing a structure quite different from those of adult animals appeared in the striated muscle of the adult rabbit after denervation This observation was later confirmed in many works in which the principal feature studied was the implantation of nerves to the previously denervated muscle (Guth and Zalewski 1963, Miledi 1963, Gwyn and Aitken 1964, 1966 Fex and Thesleff 1967 and others, cf Saito and Zacks 1969) Formation of neuromuscular connections in foetal tissue explants has also been demonstrated both in en bloc' preparations of cord somite regions (Veneroni 1968, Peterson and Crain 1968) and between separate explants of foetal mammalian tissues (Crain 1968)

The present work was performed to study the course of development of the "new', postnatally induced myoneural junctions and to discover whether morphogenetic differences exist as compared with the development of normal myoneural junctions induced in immature muscle fibres in utero and in addition to see whether the age of the animal has any effect on myoneural morphogenesis A short note on the present results has been presented in the form of an abstract (Teravainen and Juntunen 1969)

Materials and methods

In a total of 66 albino rats of Sprague Dawley strain comprising young animals of 1 5 8 10 15 days and adults of 1 to 3 months of age the sciatic nerve was compressed many times between forceps so that about 3 mm of the nerve was severed in the region of the middle third of the femur The expected re innervation of the myoneural junctions was studied from the tibialis anterior muscle at intervals of 4—7 days during 1 1/2 months after the operation

Specimens of the muscles removed under [illegible] one volume of 35 % HCHO 6 volumes of [illegible] at 4° C Frozen sections at 10 μ were cut [illegible] washed in H_2O for 1—3 hrs before the [illegible] tion of acetylcholinesterase (AChE) activity was used to study the structure of the myoneural junction with the light microscope Acetylthiocholine iodide (Fluka A G Buchs Schweiz) [illegible]

That the denervation was successful was checked histochemically by demonstrating increased ns ChE activity along intramuscular degenerating and regenerating nerves using butyrylthiocholine iodide (Fluka A G Buchs Schweiz) as substrate with 10^{-5} M 1 5 bis (4 allyl-dimethylammoniumphenyl)pentan 3-one-diiodide (284C51 Burroughs and Wellcome London) as a specific inhibitor for AChE Incubation was performed with free floating sections at 37° C for 30—120 min The sections were pre incubated for 30 min with the inhibitor before the substrate was added

Results

The muscle fibres of the normal tibialis anterior were found to contain a single connection with the motor nerve the myoneural junction being situated in a narrow zone, approximately in the middle of the fibre which greatly helped in recognizing

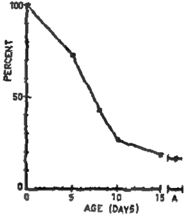

Fig 1 The number of rats in which new myoneural junctions were formed, is depicted graphically (per cent of the total number of test rats in the respective age groups

the formation of new myoneural junctions outside the innervated zone Only those myoneural junctions that were clearly separated from the zone of the previously existing myoneural junctions were considered new, provided they were not included in the intrafusal muscle fibres New myoneural junctions were observed in animals of every age group operated on but not in every individual animal, this being especially true of the older and adult animals (Fig 1) There was a considerable overlap between the successive developmental stages of individual new myoneural junctions but, as will be seen, time sequences were comparable with normal development

Rats operated on at birth New myoneural junctions were formed in each of the 10 test animals (Fig 1) They were often numerous and in some cases were distrib- [illegible] all over the muscle, although mainly proximally but sometimes also distally of the zone of old degenerating myoneural junctions (Fig 2, 3 and 4) The first new myoneural junctions were seen in animals killed 14 days after the operation. The distribution of AChE activity was plate-like (Fig 3), as in the primitive myoneural junction induced in utero during normal development (Teräväinen 1968) Axon terminals were observed to have been invaginated into the plasma membrane of the muscle fibre and the development of postsynaptic infoldings to have begun in a few of the new myoneural junctions 20 days after the operation (Fig 5) On the 30th postoperative day the axon terminals were often separated from each other, and well developed infoldings of the postsynaptic membrane were visible (Fig 6) A few well ramified new myoneural junctions with fully developed postsynaptic infoldings and almost mature appearance were seen 35 days after the operation (Fig 7)

Rats operated on at 5 days of age New myoneural junctions were found in eight of the eleven test animals The newly formed myoneural junctions were seen on the 14th postoperative day but were not so numerous as in the former group and were localized for the most part in the proximal part of the muscle The new myoneural junction was at first a plate-like structure and the postsynaptic muscle plasma membrane folded in some of them in the 20th postoperative day Ramification of the

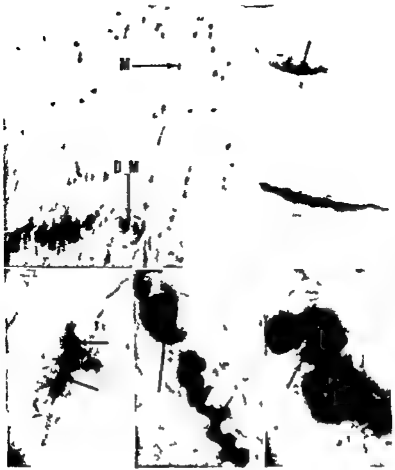

Fig 2

Fig 3

Fig 4

Fig 5 Fig 6 Fig 7

Fig 2 The figure shows new myoneural junctions (M) outside the zone of previously existing degenerating myoneural junctions (DM) in an animal denervated at birth and killed 14 days later The figure shows only the proximal part of the muscle section but new myoneural junctions were also seen in the distal part × 10

Fig 3 New myoneural junction from the proximal part of the muscle section in *Fig 2* The postsynaptic membrane is seen in side view and is completely devoid of postsynaptic infoldings (arrow) × 900

Fig 4 Degenerating old myoneural junction from the same animal as in *Fig 2* The area of AChE distribution is elongated presumably owing to growth of the muscle fibre during the regeneration period No postsynaptic infoldings were seen

Fig 5 New myoneural junction 20 days after the operation. Small postsynaptic infoldings are seen (arrows) and the area of AChE activity is wider than in *Fig 3* × 900

Fig 6 In the new myoneural junction 30 days after operation on the newborn animal relatively well developed postsynaptic infoldings are present (arrow) and some fragmentation of the area occupied by the reaction product has also occurred.

Fig 7 The distribution of AChE activity 35 days after the operation on the newborn animal reveals completely developed postsynaptic infoldings (arrow) and the myoneural structure as a whole is comparable with that of adult animals

area of AChE activity was first seen in the rats killed 30 days after the operation. New myoneural junctions with a fully developed postsynaptic structure were seen on the 35th postoperative day.

Rats operated on at 8 days of age. In three of the seven test animals new myoneural junctions were observed; these were localized exclusively in the proximal part of the muscle and at first possessed a plate like structure. Some postsynaptic infolding was seen in the specimens taken 21 days after the operation. The postsynaptic infoldings were well developed at the 36th postoperative day, when ramification of the axon terminals was also observed.

Rat operated on at 10 days of age. Altogether 11 animals were operated on, in one of which some primitive new myoneural junctions were seen in the rats killed 16 days after denervation. Myoneural junctions with primitive postsynaptic infoldings were seen in one rat 22 days after the operation, and in one animal myoneural junctions outside the innervated zone possessed a well developed postsynaptic structure 38 days postoperatively.

Rats operated on at or after 15 days of age. New myoneural junctions were seen only in 5 of the 28 test animals. The number of new myoneural junctions was very few and they were seen only sporadically in the proximal part of the muscle. After the 16th postoperative day a few primitive plate like new myoneural junctions were seen in the very proximal part of the muscle. The postsynaptic infoldings were observed 23 days after the operation; the separation of the axon terminals from each other had occurred by the 40th postoperative day and the new myoneural junction had the appearance of a fully developed normal myoneural junction.

Discussion

The present work showed that new neuromuscular junctions develop outside the innervation band during spontaneous re-innervation in the adult animal, exactly as described earlier (see Introduction).

In addition, it was shown that new myoneural junctions can be formed de novo during spontaneous re-innervation in all age groups, from newborn animals to adults, and that more new myoneural junctions are formed in young, actively developing animals than in older ones. This is probably due to mechanical factors during regeneration, which result in the escape of more fibres from the glial sheaths in animals in which growth is more rapid. In the operated animals in which no new myoneural junctions developed, the majority of the original degenerated myoneural junctions became re-innervated, thus rendering the appropriate muscle fibres incapable of forming new myoneural junctions (Csillik and Savay 1959), whilst some atrophy was seen in muscle fibres that were devoid of either new myoneural junctions or regenerated old ones.

It was also shown that the structural changes in the morphogenesis of the new myoneural junctions, beginning from the plate like structure of the postsynaptic membrane which acquires postsynaptic infoldings and later ramifies, are essentially

similar to those occurring during normal development (Teravainen 1968 Kelly and Zacks 1969) and similar in animals of all age groups The conclusion that these structural changes induced postnatally during the maturation of the myoneural junctions were similar to the events of normal myoneural development can be drawn from the fact that new myoneural junctions were found in all stages corresponding to the development of the normal myoneural junctions formed in utero Moreover, the longer the time that had passed from the operation the more mature were the new myoneural junctions observed Our observations (Teravainen and Juntunen 1969) have recently been confirmed by electron microscopic demonstration of similarities in the morphogenesis of myoneural junctions induced outside the innervation band during embryonic development (Saito and Zacks 1969)

The time sequence during the structural maturation of the new myoneural junctions is grossly similar to that occurring in normal development, secondary synaptic clefts began to form at 6—8 days ramification of the area of AChE activity took place 16—19 days after the first primitive plate like AChE positive structure was observed and mature myoneural junctions appeared at 25 to 30 days after the formation of the primitive junctions

In the present work the interval of 14—16 days between the operation and the formation of an immature new myoneural junction was an expected finding such a delay in the development of the normal myoneural junction is produced by temporary denervation of the sciatic nerve in the same site (Teravainen and Juntunen 1968) Re innervation occurred somewhat earlier in the young animals probably owing to the relatively shorter distance for the nerve to regenerate than in the older groups but when a primitive myoneural junction had been formed subsequent morphognesis proceeded identically

By demonstrating that the structural development and the time sequences between different developmental stages of the new myoneural junctions are essentially similar in all age groups and correspond in all respects to the morphogenesis induced in utero the present work indicates that the same nervous factors probably prevail in the development of both postnatally induced myoneural junctions and normal myoneural junctions induced in utero More work with different methods is needed however, to clarify the basic nature of the nervous influence

References

CRAIN S M Development of functional neuromuscular connections between separate explants of fetal mammalian tissues after maturation in culture *Anat Rec* 1968 *160* 466

CSILLIK B and G SAVAY D — Regeneration der subneuralen Apparate der motorischen End platten *Acta Neuro eg* (Wien) 1959 *19* 41 52

FEX S and S THESLEFF The time required for innervation of denervated muscle by nerve implants *Life Sci* 1967 *6* 635—639

GUTH L and A A ZALEWSKI Disposition of cholinesterases following implantation of nerve into the innervated and denervated muscle *Exp Neurol* 1963 *7* 316—326

GUTMAN E and J Z YOUNG The re innervation of muscles after various periods of atrophy *J Anat* (Lond) 1944 *78* 15—43

GWYN D G and J T AITKEN New motor end plates and their relationship to the m fiber injury *Nature* (Lond) 1964 *203* 651—652

GWYN, [redacted] G and J T AITKEN, The formation of new motor end plates in mammalian sceletal muscle *J Anat* (Lond) 1966 *100* 111—126

JUNTUNEN, J and H TERÄVÄINEN, 1969 Unpublished

MILEDI, R, Formation of extra nerve muscle junctions in innervated muscle *Nature* (Lond) 1963 *199* 1191—1192

PETERSON, [redacted] R and S M CRAIN, Re innervation of denervated mammalian sceletal muscle in vitro *Anat Rec* 1968 *160* 408

SAITO A and [redacted] I ZACKS, Fine structure of neuromuscular junctions after nerve section and inplantation of nerve in denervated muscle *Exp Molec Pathol* 1969 *10* 256—273

TERÄVÄINEN H, Carboxylic esterases in developing myoneural junctions of rat striated muscle *Histochemie* 1968a *12* 307—315

TERÄVÄINEN, H, Development of the myoneural junction in the rat *Z Zellforsch* 1968b *87* 249—256

TERÄVÄINEN, H and J JUNTUNEN, Effect of temporary denervation on the development of the acetylcholinesterase positive structures of the rat myoneural junction *Histochemie* 1968 *15* 261—269

TERÄVÄINEN, H and J JUNTUNEN Induction of motor end plates in the postnatal rat *Scand J clin Lab Invest* 1969 *23* Suppl 108

VENERONI G, Formation de novo and development of neurosmucular junctions in vitro *Anat Rec* 1968 *160* 508

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Effect of Trasylol on Thromboplastin-Induced Arterial Hypotension

By

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Abstract

NORDSTRÖM, S. *Effect of Trasylol on thromboplastin induced arterial hypotension.* Acta physiol. scand. 1970. 79. 469—474.

The effect on arterial blood pressure of pretreatment with Trasylol was studied in dogs injected i.v. with homologous brain thromboplastin. Trasylol did not consistently inhibit the initial fall in arterial blood pressure, but the return to normal pressure level was faster after administration of the drug. This effect of Trasylol might depend on interference with coagulation processes as well as with release of vasoactive substances.

Hemodynamic changes are an important manifestation of intravascular coagulation (McKay 1965, Hardaway 1966). Delin, Olsson and Teger-Nilsson (1967) have shown that thrombin and thromboplastin cause peripheral vasodilation followed by intravascular coagulation. By dialysis of tissue thromboplastin they succeeded in separating a vasodilating activity from the procoagulant activity. Nordström *et al.* (1969) found that, irrespective of pretreatment with heparin, the blood pressure decreased following i.v. injections of the thromboplastin-like procoagulant from postpartum serum. Thus thromboplastin preparations, apart from their coagulation promoting materials, contain vasoactive substances. Consequently, several mechanisms may contribute to the hypotension in thromboplastin-induced intravascular coagulation. Trasylol, which inhibits the release of vasoactive kinins (Back 1966) and acts as a weak antithromboplastin (Amris 1964, Blombäck, Blombäck and Olsson 1966) might, therefore, interfere with the thromboplastin induced hypotension.

The purpose of this investigation was to study the effect on arterial blood pressure of pretreatment with Trasylol in dogs injected i.v. with homologous brain thromboplastin. As indicators of the induced coagulation process, fibrinogen concentration and platelet count were also followed.

Materials and Methods

Dog brain thromboplastin was prepared as described previously (Nordstrom 1969) Three thromboplastin preparations with concentrations of 7—16 arbitrary units (arb u)/ml were used in the experiments

Trasylol®, a kallikrein inhibitor prepared from bovine lung containing 20 000 kallikrein inhibitory units (KIU)/ml was supplied by Bayer Farma AB Stockholm

Fibrinogen concentration platelet count and *hematocrit* were determined as described previously (Nordstrom 1969)

Experimental procedure

The experiments were performed on 13 mongrel dogs weighing between 8 and 14 kg Anesthesia was induced by i v thiopental sodium and maintained with 70 per cent dinitrogen monooxide in oxygen, administered by means of an Engstrom respirator (Mivab Sweden) When needed additional amounts of thiopental sodium were given

Polyethylene catheters were introduced through a carotid artery into the aorta and through a jugular vein into the right ventricle or the pulmonary artery and connected to the pressure recording equipment Thromboplastin was injected through a polyethylene catheter inserted from a leg vein to a central vein at the level of the heart Seven dogs received Trasylol prior to or simultaneously with the thromboplastin injection The Trasylol concentration at the time of thromboplastin injection was calculated assuming the initial half life of the inhibitory effect of i v injected Trasylol in dogs to be 30 min as in humans (Andersson Nilsson and Hedner 1967)

Blood samples were collected by venipuncture or drawn from a catheter before anesthesia and then at 5—10 and/or 15—20 min after the thromboplastin injection

The dogs were divided into the following groups (the doses of thromboplastin and of Trasylol are shown in Table I)

I *Thromboplastin injection*
6 dogs were used 4 of them were injected for 18—20 sec with undiluted thromboplastin and 2 of them for 20 sec with thromboplastin diluted in saline 1/2 and 1/4, respectively

II *Trasylol + thromboplastin injection*
7 dogs were treated with Trasylol 4 of them received the i v injection of Trasylol 2—30 min prior to the thromboplastin injection The injection time of thromboplastin was 18—20 sec The other 3 dogs were given mixtures of thromboplastin and Trasylol In the latter case thromboplastin was diluted in Trasylol saline solution 1/2—1/4 After incubation 20—30 min at room temperature the mixture was injected i v for 20 sec

Results

No significant difference in reaction pattern occurred between dogs injected with undiluted and saline diluted thromboplastin or between dogs injected with Trasylol prior to or together with thromboplastin

Effect on mean aortic blood pressure

In 2 of the 6 dogs in Group I (thromboplastin alone) and in 3 of the 7 dogs in Group II (Trasylol + thromboplastin) the aortic blood pressure increased temporarily by 10—20 per cent at the end of the thromboplastin injection During the injection, or within 10 sec after cessation of the thromboplastin injection the blood pressure fell and reached about 1 min later the lowest value (Table I) Ten min after the start of the thromboplastin injection the blood pressure had returned to 80 per cent of the preinjection value in 6 out of the 7 dogs in Group II but in none of the 6 dogs in Group I The difference is statistically significant ($0.001 < p < 0.01$) The

TABLE I Aortic blood pressure in dogs injected i v with thromboplastin alone or after pretreatment with Trasylol

Group	Dog no	Trasylol KIU/ arb u	KIU/kg	Thromboplastin arb u / kg×sec	arb u / kg	Mean aortic blood pressure (mm Hg) control	min after thromboplastin injection 1	5	10	15	20	30
I	498			0 12	2 2	102	25	36	60	64	73	87
	506			0 15	2 6	97	19	48	67	73	83	86
Thrombo-	504			0 15	2 7	125	30	77	90	115	121	
plastin	629			0 18	3 6	107	45	59	61	68	89	98
alone	635			0 20	4 1	125	22	60	60	63	104	98
	659			0 33	6 7	142	40	77	105	105	113	135
	Mean			0 19	3 7	116	30	60	72	81	97	(101)
II	511	2,500	5 000	0 12	2 1	120	30	92	95	106	109	110
	493	4 500	10,000	0 12	2 2	112	30	55	66	75	86	
Trasylol	310	25 000	65 000	0 13	2 5	89	41	70	70	115		
+	625	[illegible] 500	25,000	0 15	2 9	113	58	103	103	110	117	
thrombo-	647	2,500	10,000	0 20	4 1	127	35	68	111	109	109	111
plastin	666	1,500	10 000	0 36	7 3	106	28	66	98	83	93	
	669	3 500	30 000	0 44	8 9	118	28	66	101	104	104	
	Mean	6,900	22 000	0 22	4 3	112	36	74	92	100	(103)	

Dogs 635 and 659 were given thromboplastin diluted in saline 1/2 and 1/4 respectively Dogs 647, 666 and 669 were given thromboplastin diluted in Trasylol saline solution 1/2, 1/4 and 1/4, respectively

two dogs which were given the largest Trasylol doses per arb u of thromboplastin showed the smallest initial pressure decrease and regained the 80 per cent level within 5 min After 20 min, 5 of the dogs in Group I had reached 80 per cent of the preinjection blood pressure The changes in blood pressure are shown in Fig 1

In 2 of 4 dogs injection of Trasylol itself caused an immediate decrease in aortic blood pressure from 115 to 45 and 50 mm Hg respectively The injection rates of Trasylol were 400 and 700 KIU/kg b w and sec respectively The blood pressure returned to initial levels within 3 and 5 min respectively In the other 2 dogs the Trasylol injection started at a rate of 200 KIU/kg b w and sec and was then accelerated, in one dog up to 4000 KIU/kg b w and sec (200 000 KIU in 5 sec) without any pressure reaction

Effect on pulmonary arterial (right ventricular) pressure

Because of frequent clotting with occlusion of the catheters used for pressure recording in the pulmonary artery these catheters had to be flushed and/or moved to different positions on repeated occasions Thus reliable pressure recordings were difficult to obtain However in both groups the thromboplastin injection was followed by an increase in pulmonary arterial pressure to about double the prei[illegible]

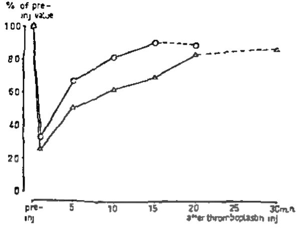

Fig 1 Mean aortic blood pressure in dogs treated with i v injection of thromboplastin alone and after pretreatment with Trasylol
△—△ thromboplastin alone, mean of 6 dogs
○—○ Trasylol + thromboplastin mean of 7 dogs

tion level This pressure increase started when the aortic pressure had reached its minimum value In both groups, the pulmonary arterial pressure returned to preinjection levels within 5 min Trasylol alone had no influence on the blood pressure in the right ventricle or pulmonary artery

Effect on fibrinogen concentration

Trasylol seemed partly to prevent the decrease in fibrinogen concentration after thromboplastin injection After the initial decrease, the fibrinogen concentration remained unchanged for at least 15—20 min in both groups (Table II)

TABLE II Simultaneous values of mean aortic blood pressure, fibrinogen concentration, platelet count and hematocrit in dogs injected i v with thromboplastin alone or after pretreatment with Trasylol Mean values (ranges) in per cent of preinjection values

		Min after thromboplastin injection 5—10	15—20
	Dogs no	498, 504, 629, 635, 659	506, 635, 659
I	Mean aortic blood pressure	60 (50—72)	80 (80—80)
Thromboplastin	Fibrinogen conc	54 (33—74)	47 (26—70)
alone	Platelet count	14 (4—23)	35 (19—4[illegible])
	Hematocrit	116 (103—133)	105 (97—119)
	Dogs no	511, 510, 625, 647, 666, 669	493, 625, 647, 666, 669
II	Mean aortic blood pressure	76 (56—91)	85 (65—97)
Trasylol	Fibrinogen conc	74 (52—106)	74 (50—113)
+	Platelet count	19 (13—36)	47 (32—65)
thromboplastin	Hematocrit	110 (97—123)	99 (82—117)

Effect on platelet count

The initial fall in platelet count after i v thromboplastin injection was about the same in both groups Like the aortic pressure, the platelet count had started to increase within 15—20 min (Table II)

Effect on hematocrit

In both groups, the hematocrit increased initially by 10—15 per cent but seemed to return to normal within 20 min

Discussion

The decrease in fibrinogen concentration and platelet count is indicative of intravascular coagulation The fall in platelet count was rapid and the lowest values were those obtained early after the thromboplastin injection The fall in arterial pressure was maximal at about 1 min after the thromboplastin injection Also the rise in pulmonary arterial or right ventricular pressure was rapid These hemodynamic changes resemble those after thrombin infusion as described by Olsson Rådegran and Taylor (1969) They suggested that such early effects are due to pulmonary vasoconstriction and systemic vasodilatation rather than to formed fibrin clots The mediators of the vascular effects may originate from the blood cells or from the plasma On the other hand later hemodynamic events may depend on clot formation

As in previous investigations on thromboplastin induced intravascular coagulation (Nordstrom 1969) pretreatment with Trasylol partly inhibited the decrease in fibrinogen concentration indicating an anticoagulant effect of Trasylol The initial fall in platelet count and in aortic blood pressure was less influenced by Trasylol the return of systemic blood pressure however being significantly faster in Trasylol treated than in untreated dogs This effect of Trasylol might be due to partial inactivation of vasoactive substances which are present in undialyzed thromboplastin The marked decrease in systemic blood pressure after rapid injections of Trasylol alone could be avoided by starting the injection at a slow rate

The conclusion is drawn that Trasylol is unable to inhibit consistently the initial fall in systemic blood pressure after i v thromboplastin injections However the return to normal pressure level is faster after administration of the drug This effect of Trasylol might depend on interference with coagulation processes as well as with release of vasoactive substances

I wish to thank Dr Erik Berglund Dr Birger Blomback and Dr Per Olsson for valuable discussions during this work and Miss Ingrid Soderman for most skilful technical assistance

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References

AMRIS C J Inhibition of fibrinolytic and thromboplastic activity by Trasylol *Abstr XI Congr Int Soc Haemat* 1966 G 72 (*Scand J Haemat* 1966 3 19—32)

ANDERSSON L, I M NILSSON and L HEDNER Experimental and clinical studies on th

fibrinolytic and presumed antithrombotic effect of a kallikrein inhibitor (Trasylol) *Scand J Urol Nephrol* 1967 *1* 11—18

BACK, N, The fibrinolysin system and vasoactive kinins *Fed Proc* 1966 *25* 77—83

BLOMBÄCK, B, M BLOMBACK and P OLSSON, Action of a proteolytic enzyme inhibitor on blood coagulation in vitro " " Eds R Gross and G Kroneberg F K Schattau

DELIN, A, P OLSSON and A the canine leg caused by intra arterial infusion of Cardiovasc Res 1967 *1* 371—378

HARDAWAY, R M, *Syndromes of disseminated intravascular coagulation* With special reference 1966

mechanism of disease

oagulation by heparin and Trasylol *Acta physiol scand* 1970 *79* 390—404

NORDSTROM, S, P OLSSON, A-C TEGER NILSSON and E ZETTERQVIST, A procoagulant from human postpartum serum Effects on coagulation and hemodynamics in dogs *Acta physiol scand* 1969 *76* 399—405

OLSSON, P, K RÅDEGRAN and G A TAYLOR, Hemodynamic changes resulting from thrombin induced intravascular coagulation 1969 In manuscript

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Temperature Regulation during Exercise Dehydration in Man

By

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Abstract

EKBLOM, B C J GREENLEAF J E GREENLEAF and L HERMANSEN *Temperature regulation during exercise dehydration in man* Acta physiol scand 1970 79 475—483

The effects of mild exercise dehydration (1 % of the body weight) on temperature regulation ... of 1 hr duration on a ... relative O_2 uptake of ... In both hydration and ... mperature (T_{re}) were 0 3° C higher than during continuous work Progressive dehydration elevated equilibrium levels of T_{re} and mean body temperatures 0 3 to 0 4° C above hydration control values while mean skin temperatures (T_s) were essentially constant at 30±0 2° C The elevation of T_{re} with dehydration was due predominantly to reduced sweating It is suggested the sweat depression was due to reduced stimuli from the central nervous system to the glands These results emphasize the sensitivity of the thermoregulatory system to dehydration and point up the need to control water intake during such experiments

When man is exposed to hot environments (>30° C T_a) dehydration results in increased core temperatures greater than those achieved at the same metabolic rate when fully hydrated both at rest (Adolph 1947 Hertzman and Ferguson 1960 Senay and Christensen (1965) and during exercise (Adolph 1947 Ladell 1955 Pitts *et al* 1944, Snellen 1966 Strydom and Holdsworth 1968) This dehydration hyperthermia increases linearly with a progressive water deficit (Adolph 1947) and exercise accelerates the rise in core temperature (Lind 1963 Schmidt-Nielsen 1964) In the environment independent zone (5 to 30° C T_a) where core temperature is determined by the level of stress (workload) and independent of drybulb temperature (Nielsen 1938 Nielsen and Nielsen 1962 Lind 1963) there is relatively little information concerning the effects of dehydration on exercise temperature regulation (Grande *et al* 1958 Monagle *et al* 1956 Rietschel and Beck 1928) The mechanism of dehydration hyperthermia has not been definitely established but appears to be due mainly to an impairment of sweating The purpose of this study was to investigate the temperature regulatory mechanisms dur

in the environment independent zone

TABLE I Individual values for various physiological variables with hydration and dehydration

Subj	$\dot{V}o_2 \cdot 100 / \dot{V}o_2$ Max %	Net Met Heat Prod kcal/(m² hr)	Tissue Conductance kcal/(m² hr °C)	ΔRectal Temp °C
Continuous work				
	hydration			
BEN	59	296	34	+1 04
BON	66	392	54	+1 78
STN	69	338	47	+1 38
$\bar{X}$	65	342	45	+1 40
	Dehydration			
BEN	60	308	42	+1 50
BON	59	321	45	+1 22
STN	63	315	44	+1 79
$\bar{X}$	61	315	44	+1 50
%Δ	−6	−8	−2	+7
Intermittent work				
	Hydration			
BEN	58	298	34	+1 77
BON	62	378	53	+1 70
STN	64	323	40	+1 46
$\bar{X}$	61	333	42	+1 61
	Dehydration			
BEN	60	316	33	+1 91
BON	60	339	48	+1 96
STN	66	346	41	+1 85
$\bar{X}$	62	334	40	+1 91
%Δ	+1	0	−5	+14
$\bar{X}$ HYD	63	338	44	+1 52
$\bar{X}$ DEHYD	61	324	42	+1 70
%Δ	−3	−4	−5	

Procedure and methods

The effects of hydration (tap water at 37° C consumed equal to water loss) and mild dehydration of about one percent of the body wt (water withheld during the one hr exercise period) on temperature regulation were studied in three male subjects (average age 23, b w 70 5 kg, surface area 1 86 m² and maximal O_2 uptake 61 4 ml/(kg min)) during continuous and during intermittent work at approximately the same average heat production on a Krogh bicycle ergometer The intermittent workload was about twice that used in continuous work and applied on a 30 sec exercise and 30 sec rest sequence that gave an average relative O_2 uptake ($\dot{V}o_2 \times 100/\dot{V}o_2$ maximum) of 62 % (Table I) The expired air was collected in Douglas bags, the volume was measured in a 150 l Tissot tank (±0 05 l) and the O_2 and CO_2 concentrations measured with a modified Haldane technique (Åstrand and Saltin 1961) During intermittent work the $\dot{V}o_2$ from the exercise and rest periods were averaged During intermittent work heart rates were averaged from the last 15 sec of a rest period and the last

ΔSkin Temp °C	ΔMean Body Temp (8T$_{re}$ + 2T$_s$) °C	ΔBody Weight g hr	Sweat Loss g (m² hr)	Respiration H_2O Loss g (m² hr)	ΔHeart Rate beats min
−2 83	+0 27	−115	369	47	71
−0 30	+1 36	−155	412	63	106
−0 95	+0 91	−24	322	67	92
−1 36	+0 85	−93	368	59	90
+1 83	+1 57	−735	318	47	80
+0 19	+1 01	−710	312	52	86
−0 32	+1 37	−755	341	58	102
+0 57	+1 32	−733	324	52	89
+339	+36	−866	−12	−12	−1
−1 82	+1 05	+35	296	45	81
−1 12	+1 13	−135	374	71	102
−1 46	+0 88	+15	293	67	78
−1 47	+1 02	−28	321	61	87
−3 03	+0 92	−690	288	50	89
−0 09	+1 55	−715	313	55	95
−1 55	+1 17	−700	274	78	107
−1 56	+1 21	−702	292	61	97
−6	+16	−960	−9	[illegible]	+10
−1 41	+0 93	−63	344	60	88
−0 50	+1 26	−718	308	57	93
			−10	−5	−5

15 sec of the succeeding work period Rectal temperature (T_{re}) was the average of measurements taken at four depths in the rectum (12 cm 17 cm 22 cm and 27 cm) with a multiple thermo couple and accurate to ±0 025° C (Nielsen 1962) Skin temperatures were measured at six locations arm, forearm dorsal hand scapula region anterior thigh and lateral leg The mean skin temperature (T_s) was the average of the six values Reference thermocouples were placed in a double thermos bottle at 48 0±0 25C Rectal temperature was measured during the 30 sec work period and skin temperature measurements followed Body wt changes were measured to ±20 g and care was taken to minimize sweat lost by dripping Sweat loss was calculated from total wt loss by subtracting an estimated insensible wt loss of 35 g/hr (Benedict and Benedict 1927) and the respiratory water loss with appropriate corrections for respiratory gas exchange Respiratory water loss (w_r) was calculated as

$$W_r = V_{E_{BTPS}} \frac{60}{Am^2}\left[\frac{\text{density } H_2O}{34\ C \text{ sat}} - \left(\frac{\text{density } H_2O}{21\ C \text{ sat}} \times \frac{\text{relative}}{\text{humidity}}\right)\right]$$

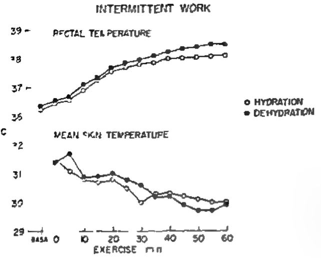

Fig 1 Comparison of rectal and mean skin temperatures with hydration and dehydration during intermittent exercise

The net metabolic heat production was the difference between the gross metabolic heat production calculated from the O_2 uptake and the heat loss due to the external work. Tissue conductance equalled heat loss from conduction and radiation plus sweat loss divided by the difference between the average T_{re} and T_s values during the last 20 min of exercise. Mean body temp (T_m = 0.8 T_{re} + 0.2 T_s). All experiments were conducted with the subjects in the basal condition. The T_a averaged 21.7 ± 0.7° C with 38% rh and turbulent air motion 120 ± 25 m/min.

Results

The average equilibrium level of intermittent work dehydration T_{re} was 38.5° C, 0.4° C higher than during hydration (Fig 1). The two T_s curves began at about 31.5° C and following essentially the same course terminated at about 30.0° C. During the last 20 min of exercise the dehydration T_s fell slightly *below* the hydration T_s (Fig 1).

During continuous work dehydration the T_{re} at 60 min was 38.1° C, 0.3° C higher than during hydration (Fig 2). The average continuous work dehydration T_s at 0 min was depressed about 1.5° C due to one subject for no apparent reason but the equilibrium level was 30.5° C, 0.5° C *above* the hydration level (Fig 1).

There was no apparent reason why both basal dehydration T_{re} were higher than their respective hydration temperatures as all three subjects were in a basal condition prior to all experiments, i.e., no food or water consumption after retiring the night before the test. The low average continuous work hydration basal T_{re} was due predominantly to one subject. However, at 15 min the hydration and dehydration T_{re} were nearly equal in the continuous and intermittent work experiments and began to separate at that point (Fig 2). It is clear that a progressive water loss to about 1% of the body weight (718 g/hr) elevates equilibrium levels of T_{re} in both continuous and intermittent exercise. Also in both hydration schemes equilibrium levels of T_{re} are higher during intermittent work (Fig 1 vs Fig 2).

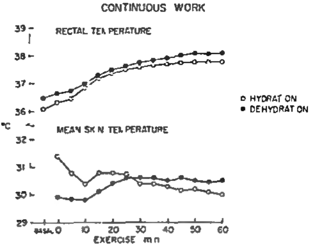

Fig 2 Comparison of rectal and mean skin temperatures with hydration and dehydration during continuous exercise

The average ΔT_{re} and Δ mean body temperatures were both elevated during dehydration (Table I). The average ΔT_{re} was 7 % greater in continuous work and 14 % larger during intermittent work. Only subject BON had a lower ΔT_{re} during continuous work. The changes in mean skin temperatures (T_s) were more variable than T_{re}. Average sweat loss was reduced in both dehydration experiments (9 % and 12 %) compared with their respective hydration values and both values in intermittent work were lower than their respective values in continuous work. Again only subject STN exhibited an increased sweating during his continuous work dehydration experiment. The average respiratory water losses were slightly depressed (5 %) with dehydration (Table I). The mean change in heart rates exhibited the well documented increase with dehydration during intermittent work but were essentially unchanged during continuous work. In both continuous and intermittent work the average tissue conductances (an estimation of the transfer of heat from the core to the periphery via the circulation) were reduced slightly (2 to 5 %) with dehydration. The hydration vs. dehydration average net metabolic heat production available for dissipation (M_{NET}) was 8 % lower in continuous work dehydration and equal during intermittent work.

In continuous work dehydration, the average reduction in sweat loss of 44 g/(m² hr) (12 %) would result in a decreased evaporative heat loss of $44 \times 0.58 =$ 26 kcal/(m² hr); the corresponding figure for intermittent work dehydration is 17 kcal/(m² hr). Assuming the specific heat of the body to be 0.83, a decreased heat loss of 26 kcal/(m² hr) would raise body temperature $(26 \times 1.86)/(0.83 \times 70.5\ \text{kg}) = 0.8°$ C in the continuous and 0.5° C in the intermittent work experiments. Thus reduced sweating and evaporative heat loss during dehydration most likely accounted for the elevated T_{re} in both modes of exercise.

In summary: (a) A progressive water loss to 1 % of the body wt elevates equilibrium levels of rectal and mean body temperatures 0.3 to 0.4°

and intermittent exercise (b) The equilibrium levels of mean skin temperature were relatively constant, 30±0 2° C (c) The elevated rectal temperatures with dehydration were due, principally, to a depression of sweating (d) In both the hydration and dehydration experiments during intermittent work, the equilibrium levels of rectal temperature were 0 3° C higher than during continuous work

Discussion

The most important result of the present study was that rectal temperature was elevated 0 3 to 0 4° C during submaximal exercise dehydration in the environment-independent zone with a total body water loss of 1 % of body weight In man, the sensitivity of the centers for temperature regulation in the brain has not been determined directly However changes in the peripheral circulation of the hand have been detected with estimated changes in brain temperature of ±0 2° C (Snell 1954) If this threshold is approximately correct, a variation in core (brain) temperature of 0 4° C could result from a decreased evaporative heat loss (dermal plus respiratory) of about 13 kcal/(m^2 hr) [22 g H_2O/(m^2 hr)] about half the decrease observed in the present study In the environment independent zone at sea level pressure respiratory water loss accounts for about 1/6 (14 to 19 %) of the total water loss under diverse exercise conditions (Greenleaf *et al* 1969, present study), sweating constitutes the remaining 5/6 of the loss Theoretically, in the absence of sweating the threshold water loss of 41 g/hr (assuming 0 03 g/l in expired air) would occur with a ventilation of 23 l/min The latter is produced at a relative O_2 uptake of 25 to 30 %

The two major divisions of the heat dissipation mechanisms during exercise between 5 and 30 C T_a involve the transference of heat from the core (working muscles) to the periphery via the circulatory system and the exchange of heat from the skin to the environment by evaporation, conduction convection and radiation Of these four mechanisms the decrease in sweating was the most pronounced (Table I) and probably contributed most to the reduction in heat dissipation and the resulting increased T_{re} with dehydration

The increased T_{re} with dehydration was not due to greater net metabolic heat production during the two dehydration experiments in fact the reverse was true (Table I) There is no doubt the cardiovascular dynamics were different during the intermittent exercise and the average dehydration tissue conductance was 5 % lower than hydration which would promote heat storage Since increased rectal temperatures with dehydration were found utilizing both continuous and intermittent work any selective result due to the nature of the exercise may be ruled out In the environment independent temperature zone the rate of sweating during exercise is proportional to the relative O_2 uptake and not to core temperature (Greenleaf *et al* 1969, Saltin and Hermansen 1966) It is not likely the 3 % decrease in the relative O_2 uptake during dehydration would cause a reduction in sweating of 10 % and lower sweat rates were not always associated with lower relative O_2 uptakes in the

individual subjects (Table I). Another possible complicating factor is that ingestion of water at body temperature usually triggers an outburst of sweating while cold water may depress sweating (Sargent 1962). Quantitative data on changes in sweating following drinking under conditions similar to those in the present study could not be found. However, Adolph *et al.* (1947) observed an increased evaporative loss of 20 g/hr in men undergoing dehydration compared with a group drinking ad libitum during the first hour of exercise in the heat. In the present study it is possible there was enhanced sweating due to drinking but it cannot be evaluated quantitatively.

Rietschel and Beck (1928) observed an average increase in T_r of 0.4° C during stair climbing in men dehydrated 1 to 2 kg of their body weight.

Grande *et al.* (1958) and Monagel *et al.* (1956), reporting data from the same basic study, also observed elevated core temperatures during exercise in the environment independent zone. Equilibrium levels of exercise T_r were recorded in 12 young men at the end of 1 hr of work on a treadmill (5.6 km/hr, 10 % grade, 25.5° C T_a and 50 % rh) before and after 5 days of restricted food (1000 kcal carbohydrate/day) and water (900 ml/day). The average T_{re} during the hydration control walks rose to 38.0° C but was elevated to 39.6° C after dehydration; the average sweat rates were 830 g/hr and 410 g/hr, respectively. The increase in T_{re} per kg of sweat depression was 1.6/0.42 = 3.8. The corresponding ratio in the present study was 0.35/0.07 = 5.0. The different dietary conditions between the present study and that of Grande *et al.* makes any quantitative comparisons impossible, but qualitatively they are in agreement.

The depression of sweating with dehydration is not caused specifically by exercise as a sweating deficit and a progressively rising core temperature also occur in subjects resting in the heat (Hertzmann and Ferguson 1960).

The question still remains how, during exercise, the sweating mechanism responds to changes in body water content. The human body is about 70 % water (50 l in a 70 kg man) and the normal daily variation in body wt is about ± 150 g (Adolph 1943). The mechanism of sweat secretion requires (a) a transfer of sufficient fluid from the extracellular spaces into the gland, (b) adequate energy to manufacture sweat of proper osmotic concentration, etc., (c) sufficient stimulation for discharge and (d) a free flow of sweat through the duct onto the skin. Regarding item (a), it is likely there was sufficient interstitial fluid bathing the sweat glands in the face of an average loss of total body water of 718 ml because (1) water shifts from the blood to the interstitium durig exercise and the volume of the extracellular fluid exhibits a very slight drop (about 200 ml) with prolonged exercise (Kozlowski and Saltin 1964) or an increase with shorter work periods (Cullumbine and Koch 1949); (2) sweating can be maintained at 2 l/hr for 2 hrs in men working in the heat (Belding and Hatch 1955), indicating the water transportation mechanism supplying the glands in the present study was operating well below maximal levels; (3) the phenomenon of "sweat gland fatigue" (a diminution in the rate) usually occurs after about 2 hours of continuous sweating (Sargent 1962); mean skin temperatures must be above 35° C and sweat depression does not occur until sweating exceeds about

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From the Department of Anatomy University of Helsinki Finland

Uptake of 5-Hydroxytryptamine by Rat Peritoneal Mast Cells in vitro

By

S E JANSSON

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Abstract

JANSSON S E *Uptake of 5 hydroxytryptamine by rat peritoneal mast cells in vitro* Acta physiol scand 1970 *79* 484—492

The mechanism of 5 hydroxytryptamine (5 HT) uptake by rat peritoneal mast cells in vitro was studied under different incubation conditions The uptake reached a saturation level even at low concentration of exogenous 5 HT resulting in a concentration ratio of more than one thousand times The uptake during the first hour was more rapid when compared with the slow uptake during succeeding hours The uptake was dependent on temperature and calcium and magnesium ions Addition of glucose to the medium did not accelerate the uptake which also was unaffected by pH alteration from 6 0 to 7 4 The proposed active uptake mechanism for 5 HT was supported by the results obtained with metabolic inhibitors those belonging to the group of uncouplers of oxidative phosphorylation effectively inhibited the uptake while sodium cyanide and sodium azide were without significant effect Results obtained further indicated that 5 HT at high exogenous concentrations may passively diffuse into the mast

Mast cells of the rat and mouse not only contain 5 hydroxytryptamine (5 HT) (Benditt *et al* 1955) but are also able to take up this amine both *in vivo* and *in vitro* (Furano and Green 1964 Eranko and Kauko 1965 Eranko and Jansson 1967) These properties are shared by neoplastic tumour mast cells (Day and Green 1960 Green and Day 1961 Day and Green 1962a 1962b Green and Furano 1962, Green 1966a 1966b Van Orden *et al* 1967) Moreover Bergendorff and Uvnas (1967) showed that isolated mast cell granules were able to take up 5 HT

Despite the well documented fact that mast cells take up 5 HT few reports are available on the mechanism of uptake Day and Stockbridge (1964) concluded in an extensive study that neoplastic tumour mast cells take up 5 HT by both an active and a passive mechanism In a previous paper (Jansson 1968) some observations were made indicating that normal mast cells also take up 5 HT by two mechanisms This is in contrast to observations on uptake of histamine which has been convincingly shown to enter normal mast cells by diffusion only (Cabut and Haegermark 1966) The present paper deals with the mechanism of 5 HT uptake by normal mast cells *in vitro*

Materials and methods

Rat peritoneal cells of which mast cells constituted 3 per cent (Jansson 1967) were used in the present study After decapitation of the rat (Sprague Dawley) anesthetized with ether the peritoneal cells were washed out with 10 ml of an ice-cold modified Krebs Ringer solution supplemented with glucose and calcium chloride (Eränko and Räisänen 1966) The prepara tive procedures were continued as earlier explained (Jansson 1969) The incubations were performed in ll ml siliconized culture tubes in a water bath Before incubation the cells were pre incubated for about 15 min at the incubation temperature Thereafter 1 ml of the 5 HT

tion at 2500×g for 10 min The cell pellet was then resuspended in two changes of fresh Krebs-Ringer solution to wash out amine adsorbed on the cells and these were again col lected by centrifugation The 5 HT content of the washed cell pellet was determined according to the spectrophotofluorometric method of Weissbach (1961) as earlier explained (Jansson 1969)

Reagents Reserpine (Medica Oy, Ab), imipramine (Medica Oy Ab) chlorpromazine (Medica Oy Ab), mepyramine (Orion Oy), prenylamine (Segontine® Hoechst Ag) serotonin creatinine sulphate (Fluka Ag) The 5 HT concentrations refer to the base

Results

Effect of the incubation time

At high concentrations of 5 HT (4 4 μg/ml) the uptake continued to increase with the incubation time for 4 hrs (Fig 1) The uptake during the first hour was of the same magnitude or a little higher than the uptake at a 10 times smaller 5-HT con centration At low concentrations (0 44 or 0 22 μg/ml) a rapid uptake during the first hour was followed by a slight and slow uptake during the succeeding hours (Fig 4, controls, solid circles and circles with asterisk)

Effect of the 5 HT concentration in the medium

5 HT was taken up even from low concentrations (Fig 2) The uptake increased with increasing concentrations up to about 0 5 μg/ml but increasing the concen tration from this value did not further increase the uptake during 1 hr The average uptake of 5 HT at the exogenous concentration 0 44 μg/ml of the 4 expts in Fig 2

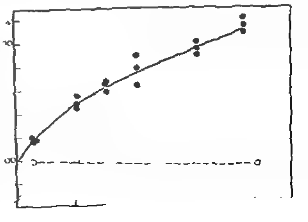


Fig 1 Uptake of 5-HT by mast cells in vitro as a function of time The 5 HT concentration in the medium was 4 4 μg/ml and the incubation temperature 37° C Each point represents the result of 1 expt. Abscissa Incubation time in hours Ordinate Mast cell 5-HT in per cent of control The 5 HT content (μg/10^6 mast cells) of samples taken at zero time and set at 100 % served as basis when the uptake was calcu lated ● Cells incubated with 5-HT ◘ control cells incubated without 5-HT

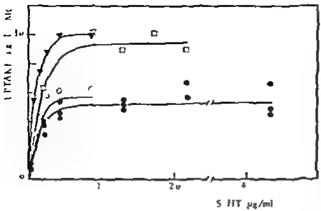


Fig 2 Uptake of 5 HT by mast cells in vitro as a function of the 5 HT concentration in the medium The incubations were carried out for 1 hr at 37° C Each curve represents 1 expt and the points the individual results in each experiment Abscissa 5 HT concentration in the medium in µg/ml Ordinate Uptake of 5 HT in µg/10^9 mast cells (MC) The uptake is the differences in the 5 HT content after incubation in the presence of 5 HT and without 5-HT but otherwise under similar conditions

was 0 72 µg/10^9 mast cells which gives a concentration ratio of 1600 after incubation for 1 hr at 37° C assuming that the volume of 10^9 mast cells is 1 µl When the concentration in the medium (Co µg/ml) was plotted against the concentration in the medium divided by the initial rate of uptake (Ci increase in mast cell 5 HT in 1 hr at 37° C µg/10^9 mast cells/1 hr) with concentrations up to 0 44 µg/ml the points fell on a fairly straight line (Lineweaver and Burk 1934) (Fig 3)

Effect of the temperature

At 0 C practically no 5 HT was taken up at an external 5 HT concentration of 0 44 µg/ml At the same concentration at 23° C the uptake was as rapid as at 37° C during the first 15 min but no further uptake was observed during the succeeding hours the 5 HT level remaining about 35 % higher than that of the control (Fig 4) At lower concentrations of 5 HT in the medium (0 22 µg/ml) no uptake was observed at 23° C

Effect of pH

The uptake of 5 HT was not affected by pH alterations from 6 0 to 7 4 but a suggestive decline was observed at pH 7 5 (Fig 5) Further experiments at higher pH resulted in turbidity of the incubation medium

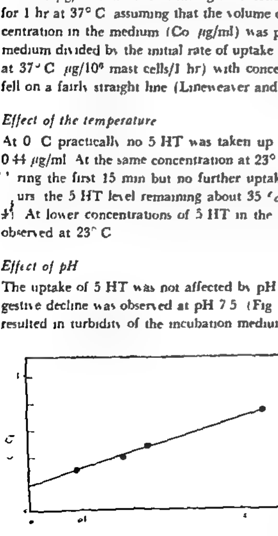
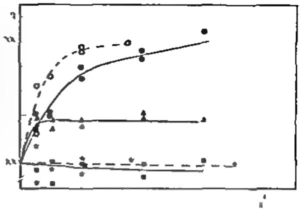


Fig 3 Relation between the initial 5 HT concentration in the medium in µg/ml (Co) and the initial rate of uptake of 5 HT by mast cells in vitro The initial rate of uptake was defined as the increase in 5 HT content of the mast cells after incubation for 1 hr at 37° C at exogenous 5 HT concentrations up to 0 44 µg/ml (Ci µg/10^6 mast cells/hour) Abscissa 5 HT in the medium (Co) Ordinate 5 HT in the medium/initial rate of uptake Co/Ci

Fig 4 Uptake of 5 HT by mast cells in vitro as a function of temperature The incubations were carried out for different lengths of time at 37° C, 23° C and 0° C, respectively The 5 HT concentration was 0 44 μg/ml and 0 22 μg/ml respectively Each point represents the result of 1 expt Abscissa Incubation time in hours Ordinate Mast cell 5 HT in percent of control ● Incubation performed at 37° C at 0 44 μg/ml ▲ Incubation performed at 23° C at 0 44 μg/ml ■ Incubation performed at 0° C at 0 44 μg/ml Incubation performed at 37° C at 0 22 μg/ml Incubation performed at 23° C at 0 22 μg/ml



Effect of calcium and magnesium ions

Calcium and magnesium ions had no great effect on the uptake at the concentrations tested (from 0 to 2 2 and 3 6 mM respectively) However, when both ions were omitted from the medium the uptake decreased to about one third of the amount taken up with the ions present (Fig 6

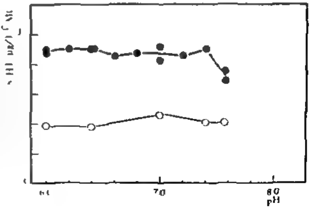
Effect of sugars

Glucose, at a concentration of 5 mM did not significantly affect the uptake (87 3 %) as compared to the uptake without glucose 95 7 %) Neither did other carbohydrates tested (galactose sucrose lactose fructose xylose ribose) have any greater effect on the uptake as compared with the uptake in the presence of glucose

Effect of metabolic inhibitors

Of the metabolic inhibitors tested only dinitrophenol and FCCP[1] (at 10⁻⁴ M and

Fig 5 Effect of pH on the uptake of 5 HT by mast cells in vitro The different pH values were achieved by varying the composition of the phosphate buffer The incubations were carried out for 1 hr at 37° C at 0 44 μg/ml Each point represents the result of 1 expt Abscissa pH of the incubation medium in pH units Ordinate Mast cell 5 HT in μg/10⁶ mast cells (MC) ○ Control cells not incubated with 5 HT ● 5 HT incubated cells



[1] FCCP carbonyl cyanide p trifluoromethoxy phenylhydrazone was obtained from M Wikstrom Dept of Clinical Chemistry Meilahti Hospital Helsinki as a sample of a gift from P G Heytler

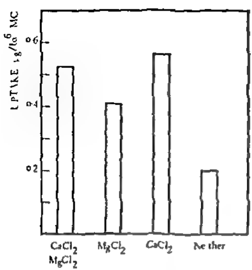


Fig [illegible] Effect of calcium and *magnesium chloride on the uptake* of 5 HT by mast cells in vitro The incubations were carried out at 0 44 μg/ml 5 HT for 1 hr at 37° C in Krebs Ringer solution supplemented with calcium and magnesium chloride (control), either with one of these salts or without them Means of 6 expts

10^{-6} M, respectively) effectively depressed the uptake of 5 HT (Table I) Sodium cyanide had only a slight depressing effect at the concentration 10^{-4} M which did not correlate to the incubation time nor to the 5 HT concentration in the medium

Effect of endogenous amounts of 5 HT

The uptake of 5 HT showed no correlation to the level of 5 HT in the mast cells within the range tested but the uptake varied much from experiment to experiment In the present study the mean uptake of 5 HT at a 5 HT concentration in the medium of 0 44 μg/ml during 1 hr at 37° C was 0 58 (standard deviation 0 07) μg/10^6 mast cells on incubation in Krebs Ringer solution supplemented with glucose and calcium chloride

TABLE I Effect of metabolic inhibitors on the uptake of 5 HT by mast cells in vitro

Inhibitor	Concentration	Number of tests	Inhibition of 5 HT uptake %
NaCN	10^{-4} M	3	22 2
NaN_3	10^{-4} M	3	18 3
H_4ClNO	10^{-4} M	3	17 1
DNP	10^{-4} M	[illegible]	63 1
FCCP	10^{-6} M	3	98 7

NaN_3 = sodium azide H_4ClNO = hydroxylammonium chloride DNP = dinitrophenol FCCP = carbonyl cyanide p trifluoromethoxy phenylhydrazone

The effect of the inhibitors is expressed in per cent using the uptake of 5 HT at 0 44 μg/ml without any inhibitor present as basis

TABLE II Effect of drugs on the uptake of 5-HT by mast cells in vitro

Drug	5-HT content after pre incubation with drug % of original value	Drug induced inhibition of 5 HT uptake, %
Prenylamine, 10^{-5} M	−5.4	122.6*
Chlorpromazine, 10^{-5} M	+6.7	98.7
Mepyramine, 10^{-3} M	−14.2	131.9**
Reserpine, 10^{-4} M	+3.8	83.0

* complete inhibition 5-HT content 22.6 % under original level

** complete inhibition 5-HT content 31.9 % under original level

After pre incubation with the drug for 15 min at 37° C the effect of the drug on the endogenous 5-HT content was determined 5-HT at 0.44 µg/ml was then added and the incubation was continued in the presence of the inhibitor drug for 1 hr at 37° C The inhibiting effect of the drugs is expressed in per cent using the control uptake as basis Means of 2 expts

Effect of drugs

Table II shows the inhibiting effect of some drugs on the 5 HT uptake The concentrations of the drugs, prenylamine chlorpromazine, mepyramine and reserpine were chosen to have no great effect on the endogenous 5-HT content during the pre incubation In the few experiments carried out in this way, with the inhibitor drug present during the whole 5 HT incubation, all drugs tested significantly depressed the 5-HT uptake It was observed that the drugs were effective in inhibiting the uptake regardless of the lengths of the pre incubation and the succeeding 5-HT incubation

The effect of imipramine and reserpine is shown in more detail in Fig 7 In these experiments the cells were incubated in the presence of the drugs at 10^{-5} M for half an hour at 37° C Thereafter the cells were spun down the supernatant discarded

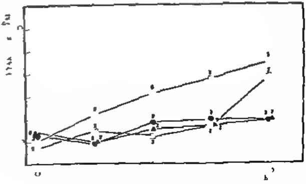


Fig 7 Effect of pre-incubation with reserpine or imipramine on the uptake of 5 HT by mast cells in vitro The cells were pre incubated with reserpine (10^{-5} M) or imipramine (10^{-6} M or 10^{-5} M respectively) for 30 min at 37° C Thereafter the cells were spun down and resuspended in fresh Krebs-Ringer solution The points are means of a number of experiments expressed by figures at various points on the curves Abscissa Incubation time in hours Ordinate Uptake of 5-HT in µg/10^6 mast cells ○ Control + 5-HT ▲ Reserpine 10^{-5} M + 5-HT ● Imipramine 10^{-5} M + 5 HT □ Imipramine 10^{-6} M + 5 HT

and the cell pellet resuspended in 1 0 ml of fresh Krebs Ringer solution 5 HT was added after about 15 min to let the cells achieve the incubation temperature Under these mild conditions both drugs depressed the uptake by keeping it on a fairly constant level achieved after incubation for half an hour After 2 hrs incubation the uptake was still considerably less than 50 % of the control uptake without inhibitor

Discussion

According to the results of the present study the uptake of 5 HT by normal rat peritoneal mast cells shows the following features (1) 5 HT is taken up from low concentrations in the medium resulting in a concentration ratio of more than 1000 times (2) The results fit the Michaelis Menten equation when the initial rate of uptake is plotted against the concentration in the medium (3) The uptake is highly temperature dependent (4) A saturation level is achieved even at low concentrations of 5 HT in the medium (5) The uptake is inhibited by reserpine and imipramine (6) The uptake is depressed when the calcium and magnesium ions are omitted from the medium

These phenomena can be taken to suggest that there is an active component in the uptake of 5 HT by mast cells a component requiring energy from cellular metabolism This view is supported by the results obtained with the metabolic inhibitors of which those belonging to the group of uncouplers of oxidative phosphorylation (dinitrophenol FCCP see Lehninger 1964) effeticeevly depressed the uptake If the uptake process is dependent on ATP it is also understandable that inhibitors such as ...um cyanide and sodium azide which in contrast to uncouplers of oxidative ...sphorylation only inhibits the regeneration of ATP leaving pre formed ATP ... d had no significant effect on the uptake Inhibition experiments with both oligomycin and dinitrophenol simultaneously in the medium might have demonstrated the specific ATP effect on the uptake because oligomycin specifically inhibits the mitochondrial ATPase activity which is induced by uncouplers of oxidative phosphorylation (Lardy et al 1958) Unfortunately oligomycin (10 µg/ml) was found to be a potent 5 HT liberator emptying mast cells of 5 HT almost completely in 1 hr why such experiments could not be carried out When it is thus indirectly demonstrated that the endogenous mast cell ATP is sufficient for the uptake mechanism to function it also seems clear that the addition of glucose or other carbohydrates have no effect on the uptake although it has been demonstrated that at least glucose fructose and galactose are taken up and oxidatively utilized by mast cells (Chakravarty 1968

An active uptake mechanism for 5 HT in the mast cells is further suggested by the work of Stockbridge and Day 1964) who found that neoplastic tumour mast cells take up 5-HT by a mechanism requiring energy They further demonstrated that 5 HT was also taken up by diffusion i e by a passive mechanism In the present work the main interest was focused on a presumptive active mechanism It was observed however that when incubating at high concentration of 5 HT for many

hours the uptake did not reach any saturation level but continued to increase with the incubation time, reaching after 4 hrs a level more than 200 % above the original 5-HT level This suggests that a passive diffusion of 5 HT takes place into normal mast cells as well This view is also supported by earlier results (Jansson 1968)

It has long been a matter of interest whether amines taken up by mast cells during incubation are stored exclusively in the granules or also in the cytoplasmic extra granular pool Furano and Green (1964) have found that additional 5 HT can be found in the cytoplasm of neoplastic mast cells if granular binding sites are saturated while Thon and Uvnas (1966) found that all histamine taken up by normal mast cells is bound to the granules My own preliminary observation suggest that 5 HT taken up is also rapidly bound to the granules in normal mast cells Further work to solve this question is in progress

References

BENDITT, E P, R L WONG M ARASE and E ROEPER 5-Hydroxytryptamine in mast cells. *Proc Soc exp Biol* (N Y) 1955 *90* 303

BERGENDORFF A and B UVNAS Uptake of biogenic amines by mast cells granules *Acta pharmacol* (Kbh) 1967 25 Suppl 4 32

CARLT M and Ö HAEGERMARK Uptake storage and release of histamine by rat peritoneal mast cells in vitro *Acta physiol scand* 1966 *68* 206

CHAKRAVARTY N Uptake and oxidative utilization of glucose fructose and galactose by rat mast cells *Biochem Pharmacol* 1968 *17* 643

DAY S M and J P GREEN Amine metabolism in mast cells *Pharmacologist* 1960 *2* 68

DAY M and J P GREEN The uptake of biogenic amines by neoplastic mast cells in culture *J Physiol* (Lond) 1962 a *164* 227

DAY S M and J P GREEN Differences in turnover of endogenous and exogenous 5 hydroxy tryptophan 5 hydroxytryptamine and histamine in neoplastic mast cells in culture *Biochem Pharmacol* 1962 b *11* 1043

DAY M and A STOCKBRIDGE The effect of drugs on the uptake of amines by mast cells *Brit J Pharmacol* 1964 *23* 405

ERÄNKÖ O and S E JANSSON Uptake of monoamines by mouse peritoneal mast cells in vitro *Acta physiol scand* 1967 *70* 449

ERÄNKÖ O and L KALKO Uptake of monoamines by mesenteric mast cells of the mouse *Acta physiol scand* 1965 *64* 283

ERÄNKÖ O and L RAISANEN *In vitro release and uptake of noradrenaline in the rat iris* In Mechanisms of Release of Biogenic Amines Eds U S von Euler S Rosell and B Uvnas Pergamon Press Oxford 1966

FURANO A V and J P GREEN The uptake of biogenic amines by mast cells of the rat *J Physiol* (Lond) 1964 a *170* 263

FURANO A V and J P GREEN The compartmentation and elimination of ^{14}C-histamine by neoplastic mast cells in culture *Biochem Biophys Acta* Amst 1964 b *86* 396

GREEN J P [illegible]
of neopla[illegible]

GREEN J P [illegible]
In Mec[illegible]
B Uvnas Pergamon Press Oxford 1966 b

GREEN J P and S M DAY Uptake and metabolism of amines by mast cells in culture *Fed Proc* 1961 *20* 136

GREEN J P and A V FURANO Two pools for amines in neoplastic mast cells *Biochem Pharmacol* 1962 *11* 1049

JANSSON S E, Effect of pH and temperature on the uptake of monoamines by mouse peri[illegible] *[illegible]and* 1968 *73* 196

[illegible] [illegible]ptamine prenylamine and reserpine on 5 hydroxy[illegible] rat peritoneal mast cells incubated in vitro *Acta*

Jansson, [redacted]-E, B Kock and O Wegelius, Separation of mast cells from the peritoneal fluid of the rat with a GE Nucleopore filter *Experientia* (Basel) 1967 *23* 407

Lardy, H A, D Johnson and W C McMurray, A survey of toxic antibiotics in respiratory phosphorylative and glycolytic systems *Arch Biochem* 1958 *78* 587

Lehninger, A L, *The mitochondrion* W A Benjamin Inc New York Amsterdam 1964

Lineweaver H and D Burk, The determination of enzyme constants *J Amer chem Soc* 1934 *56* 658

Thon, I L and B Uvnäs, Mode of storage of histamine in mast cells *Acta physiol scand* 1966 *67* 455

Van Orden, L S, I Vugman, K [redacted] Bensch and N J Giarman, Biochemical, histochemical and electron microscopic studies of 5 hydroxytryptamine in neoplastic mast cells *J Pharmacol exp Ther* 1967 *158* 195

Weissbach H, The estimation of serotonine in biological material *Stand Meth Clin Chem* 1961 *4* 197

Acta physiol scand. 1970. 79. 493—505.

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Effects of Reptilase-Induced Intravascular Coagulation in Dogs

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Abstract

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The [illegible]

fibrinolytic system was demonstrated by the appearance in plasma and serum of fibrinogen degradation products, studied by radioactivity measurements and immunological methods Epsilon [illegible]

ab [illegible]

co [illegible]

was maintained undisturbed probably due to the fact that the platelet count was essentially unchanged

It is well known that a number of enzymes, in addition to thrombin possess the capacity of transforming fibrinogen to fibrin (Eagle 1937, Eagle and Harris 1937) It has been shown that papain and also ficin clot fibrinogen (Eagle and Harris 1937, Steiner and Laki 1957)

Venoms from a number of snakes accelerate the clotting of blood The clot promoting agent has been purified from the venoms of *Bothrops jararaca* and *Bothrops atrox* (von Klobusitzky 1935 von Klobusitzky and König 1936) The compound named Reptilase has been found to have a thrombin like activity on fibrinogen and on tosylarginine methylester (Blombäck Blombäck and Nilsson 1957 Hohnen 1957) Like papain and ficin Reptilase is not inhibited by heparin even in the presence of heparin co-factor and no inactivation by the progressive antithrombin in plasma occurs Reptilase showed no fibrinolytic activity at the concentrations used for

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clotting of fibrinogen (Blomback *et al* 1957) The clots formed by Reptilase have macroscopically the same appearance as those formed by thrombin N terminal amino-acid analysis of such clots from bovine fibrinogen as well as analysis of the clot supernatant showed that only fibrinopeptide A was released during clotting (Blomback 1958, Blomback and Laurent 1958) Studies of fibrin polymerization by means of light scattering indicated that Reptilase fibrin initially polymerized mainly end to end, whereas thrombin fibrin initially polymerized also side to side In view of these findings Laurent and Blomback (1958) suggested that the release of fibrinopeptide B might facilitate the side to side polymerization The venom of the Malayan pit viper, *Agkistrodon rhodostoma,* has also been demonstrated to have a thrombin-like activity (Chan 1964, Chan, Rizza and Henderson 1965, Reid 1966) Like Reptilase, the Malayan pit viper venom is not inhibited by heparin in plasma

In the present investigation the effect of Reptilase on platelets and plasma fibrinogen was studied in dogs The influence of heparin and fibrinolytic inhibitors on the Reptilase induced intravascular coagulation was also investigated In addition results of long term treatment by daily injections of Reptilase for several weeks are reported

Materials

Dog fibrinogen was purified from fraction I—0 (Blomback and Blomback 1956) by the dimethylformamide (DMFA) method (Blomback Blomback and Holmberg 1966 Blomback and Blomback 1966) The same batch of fibrinogen was used throughout the experiments It had a coagulability of 98 %

Reptilase R was obtained from Pentapharm Ltd Basel as freezedried preparations containing the active principle together with stabilizers to a total amount of 34 mg per ampoule Compared to bovine thrombin the preparation had a clotting activity equivalent to about 0 1 NIH[1] units mg of total substance

Heparin 5000 ... *Ea lon am no*
aproic ac
Trasylol®
ester hydr
For Ra
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Germany *Triton X 100* Pacard Instr Comp Inc Downers grove Ill USA *Soluene* Pacard Instr Comp Inc Downers grove Ill USA

For Immunological test *Special Agar Noble* Difco Laboratories Detroit Michigan USA *Barbital buffer* ionic strength 0 1 pH 8 6 LKB products Stockholm *Antiserum* rabbit anti dog fibrinogen serum was prepared as previously described by Zetterqvist (1969)

Methods

... IMS-3)
... labelled
... $cm^2 \times$
... ibrated
with 0 055 M ... labelled protein
emerged with ... d
with ethanol ... a
final concentration ... n
was lowered to $-3°$ C After the addition of ethanol the solution was left standing over night Precipitated protein was removed by centrifugation and thereafter redissolved in trisodium

[1] National Institutes of Health

citrate (0 055 M pH 6 8) at 30° C to a final protein concentration of about 7 7 mg/ml (range 2 3—10 8 mg/ml) The different preparations of labelled fibrinogen had a coagulability of about [illegible] % (range 79—93) and a specific activity of 1 7 μC/mg (range 1 4—2 [illegible] μC/mg) The labelling was on the average 1 05 (range 0 7—1 9) atoms of iodine per mole of fibrinogen

Radioactivity counting The radioactivity measurements were performed in a liquid scintillation counter (Beckman CPM-200) To 0 5 ml of serum or plasma (if necessary diluted in physiological saline) was added 1 5 ml Soluene dioxan (1 part Soluene 1 part dioxan) and the mixture was heated to 60° C for 1 hr Afterwards 10 ml of scintillation liquid (7 g PPO dissolved in 1000 ml of a mixture consisting of 1 part Triton X 100 and 2 parts toluol) was added and the samples were then stored in darkness for 24 48 hrs at +4° C before counting Washed clots from [illegible] 2—0 5 ml of plasma were wound up on glass rods and dissolved in 1 5 ml Soluene-dioxan mixture, whereupon 0 5 ml of physiological saline was added The further procedure was performed as described above Error in counting exceeding 5 % was not accepted Exception was made for a few samples with a radioactivity less than 0 9 10 [illegible] μC/ml where a counting error of 10 % was accepted

[illegible]

after [illegible] and 48 hrs

Fibrinogen determinations were performed according to Bergstrom Blomback and Kleen (1960) Plasma from blood samples containing TAMe (6 μmoles/ml blood) were clotted at a thrombin concentration of 20 NIH/ml instead of the recommended 5 NIH/ml In control experiments however, TAMe was not found to interfere with the test system even at a thrombin [illegible]

[illegible] nd Kleen (1960) [illegible] with and without

[illegible]

Prothrombin was determined with a lyophilized prothrombin-converting reagent (Coatest) pre[illegible] 1965

[illegible]

the increase in serum radioactivity

Blood sampling procedure Citrated blood samples were drawn (1 part 0 1 M trisodium [illegible]

[illegible]

infusion The pH of the plasma samples was not significantly affected by the added amount of TAMe

Serum samples were obtained after spontaneous clotting of blood in glass tubes for [illegible] hrs In blood samples where heparin was present further clotting was achieved by adding 0 1 ml of thrombin (100 NIH units/ml) to 0 9 ml of sample and the tube was allowed to stand for another 2 hrs In order to prevent *in vitro* fibrinolysis EACA was added to a concentration of 2—8 mg per ml of blood in all sampling tubes

Experimental Procedure

[illegible] dogs 8 5—20 kg in weight were used In order to reveal the dose response and influence on blood pressure, pulse rate and body temperature, preliminary studies were performed on the dogs The other twelve dogs were given ^{125}I labelled fibrinogen intravenously 2 prior to the experiment The dogs were given on the average 3 [illegible] (range [illegible] 4—7 5)

TABLE I Experimental groups Simultaneous treatment (+ additional doses)

Group	Number of dogs	Reptilase mg/kg b w	Heparin IU/kg b w	EACA g/kg b w	Trasylol KIU × 10 ³/kg b w
I	3	1 7			
II	3	1 7	500 (+500+500)		
III	3	1 7		0 8 (+0 4+0 4)	
IV	3	1 7			100 (+50+50)
V	2	1 7	long term treatment, see text		

1 7 mg of Reptilase corresponds to 0 19 NIH unit of thrombin In one dog in each of Groups II (Dog 922), III (Dog 871) and IV (Dog 927), additional doses of heparin, EACA or Trasylol were given 2 and 4 hrs after the start of the Reptilase infusion One of the dogs in each of Groups III (Dog 871) and IV (Dog 837) was given further treatment with Reptilase and, consequently, after the first day referred to Group V

b w The initial plasma radioactivity was 70 5 × 10 ³ μC (range 34 5—105 5 × 10 ³ μC) per ml Potassium iodine (0 5—1 0 g) was given daily to the dogs from 1—3 days before injection of the labelled fibrinogen

Before the experiment the dogs were anesthetized by i v injection of thiopenthal sodium All infusions were given by means of an infusion pump The Reptilase was infused for 1 hr through a polyethylene catheter into a central vein or the right atrium Other drugs (or physiological saline) were infused through a separate catheter into a peripheral vein Blood samples were collected immediately before the infusion of Reptilase and then at 1/2, 1, 2, 4, 6 and in several dogs at 8 hrs after the start of the infusion

The dogs were divided into 5 groups as shown in Table I

In group V, after the initial Reptilase infusion of 1 7 mg per kg b w two dogs (no 837 and 71) were given one daily injection of Reptilase for the next 10 and 16 days, respectively In [illegible]

Results

In preliminary experiments Reptilase in doses up to 7 7 mg (corresponding to 0 86 NIH units of thrombin) per kg b w and hr did not influence arterial blood pressure, pulse rate or rectal temperature Complete fibrinogen depletion was achieved within 2 hrs after the largest dose and after 4—6 hrs after a dose of 3 4 mg per kg b w Infusion of 0 9 mg Reptilase per kg b w caused a decrease to about 60 per cent of the pre-infusion fibrinogen concentration 6 hrs later The platelet count did not decrease below 40 per cent of the pre infusion value after doses up to 7 7 mg per kg b w and hr

Factor V, tested in one dog given 1 7 mg of Reptilase per kg b w decreased during the first hr by 50 per cent and during the next 7 hrs further by 25 per cent of the pre-infusion value Prothrombin tested in 2 dogs given 1 7 mg per kg b w, was not significantly influenced in one and decreased by 30 per cent in the other dog No increased fibrinolytic activity in plasma was found after Reptilase infusion when tested in [illegible] dogs

TABLE II Effects of Reptilase in dogs untreated (I) and pretreated with heparin (II), EACA (III) or Trasylol (IV) Mean values of 3 dogs in each group

	Group	Control before exp	Time after start of Reptilase infusion hrs					days
			1/2	1	2	4	8	
Fibrinogen g 100 ml	I	0 37	0 35	(0 30)	(0 22)	0 11	(0 07)	0 06
	II	0 57	0 56	0 52	0 37	0 27	0 17	0 07
	III	0 51	0 46	(0 35)	(0 18)	0 11	(0)	(0 10)
	IV	0 52	(0 50)	(0 39)	0 30	0 17	0 07	(0 11)
Platelets 10^3/mm^3	I	450	403	319	329	339	336	325
	II	381	376	374	371	350	315	349
	III	341	420	425	305	302	(341)	(268)
	IV	363	313	315	323	342	295	231
NCFP I g 100 ml	I	0	0 01	(0 0?)	0 08	0 10	(0 06)	
	II	0	0 01	0 02	0 06	0 11	0 14	
	III	0	0	(0)	(0 06)	0 08	(0 09)	
	IV	0	(0)	(0)	(0 04)	0 06	0 02	
NCFP II g 100 ml	I	0	0	0 01	0 08	0 08	0 07	
	II	0	0 04	0	0 05	0 08	0 13	
	III	0	0	0 01	0	0	0 01	
	IV	0	0	0	0	0 0?	0 03	

NCFP I non clottable fibrinogen degradation products calculated from plasma and fibrinogen radioactivity
NCFP II non-clottable fibrinogen degradation products calculated from serum radioactivity
Values in ( ) means one sample missing

In 8 of the dogs, injected with the isotope 4—5 days prior to the experiment the biological half life of labelled fibrinogen was calculated graphically from the radioactivity values of the last 3 samples taken before Reptilase infusion The mean value was 2 2 days and the range 1 6—2 8 days The results are in agreement with those of previous studies (Nordstrom and Zetterqvist 1968 1969)

The hematocrit rose in EACA treated dogs to a value of about 20 per cent above the initial level In the other dogs the hematocrit decreased by 5—15 per cent All plasma and serum values were, therefore corrected with regard to changes in hematocrit (Nordstrom and Zetterqvist 1969)

Group I Reptilase

The dose of 1 7 mg of Reptilase caused a decrease in fibrinogen concentration and platelet count (Table II) This indicates that Reptilase induces a coagulation process Complete fibrinogen depletion was obtained in only one dog On the first day after the experiment the fibrino

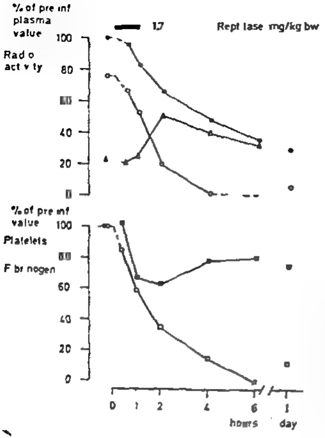

Fig 1 Infusion of Reptilase in Dog No 865 Plasma radioactivity before experiment 34 1 10^{-3} μC ml. Radioactivity in plasma ●—● fibrinogen ○—○ and serum △—△ Platelets ■—■ Fibrinogen concentration □—□

dogs The pre infusion fibrinogen value was reached on the third day in one and still later in the other dogs The platelet count fell maximally by 35 per cent.

Fig 1 shows the immediate decrease in plasma and fibrinogen radioactivity after administration of Reptilase After 1 hr the curve for plasma radioactivity levelled off and the radioactivity in serum started to increase This is in conformity with the immuno-electrophoretic investigations which showed that precipitation arcs appeared in the 1 hr sample (Fig 2 a The precipitation bands are localized in the regions of the β-globulin and the α-globulin where the lytic fibrinogen and fibrin degradation products are to be found according to Nussenzweig Seligmann and Grabar (1961) In the samples taken 2—6 hrs after the start of the Reptilase infusion the precipitation bands were more pronounced In the 24 hrs sample however they had almost completely disappeared The immunodiffusion investigation gave the same results (*cf* Fig 3 a) It is obvious that the unclottable degradation products containing the antigenic determinants were brought back to the circulation secondary to the defibrinogenation process

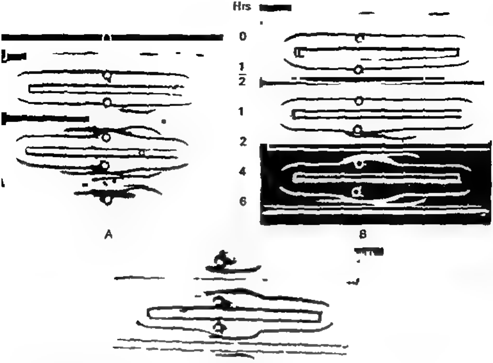

Fig 2 Immunoelectrophoresis of consecutive serum samples before and after infusion of *A* Reptilase (Dog 865) and *B* Reptilase and EACA (Dog 809)
Circular holes ——— serum samples
long basins ——— antiserum
short basins ——— fibrinogen
C. Control The circular basins contain dog fibrinogen

Group II Heparin and Reptilase

Heparin did not significantly inhibit the Reptilase induced decrease in fibrinogen concentration The platelet count was essentially unaffected (Table II) which differs from the reaction pattern in unheparinized dogs The radioactivity curves were mainly in agreement with those obtained in unheparinized dogs (Fig 4) The fibrinolytic response was also similar to that of the dogs in Group I as judged from the immuno electrophoretic (*cf* Fig 2 a) and immunodiffusion experiments (Fig 3 a) and from the radioactivity values (Table II) In some dogs there was a disagreement between the results of different methods for calculation of NCFP This might be due to occlusion of labelled degradation products in the clots during spontaneous clotting or to errors of the determinations

Group III EACA and Reptilase

Two of the dogs were completely defibrinogenated within 4 and 6 hrs, respectively The platelet count was initially increased, but decreased after 2 hrs to slightly below the initial value (Table II) The radioactivity curves for plasma and fibrinogen did

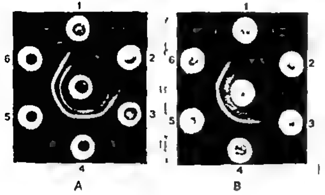

Fig 3 Immunodiffusion of consecutive serum samples before and after infusion of *A* Reptilase and Heparin (Dog 922) and *B* Reptilase and EACA (Dog 809)
1 before 2 1/2 hr 3 1 hr 4 2 hrs 5 4 hrs 6 6 hrs The central hole contains antiserum

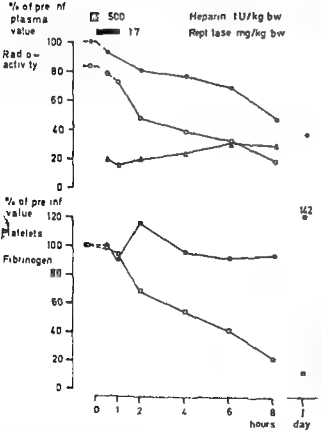

Fig 4 Reptilase infusion after heparinization, Dog No 836 Plasma radioactivity before experiment 21 6 10 ^{3} μC/ml Radioactivity in plasma ●—● fibrinogen○—○ and serum△—△ Platelets ■—■ Fibrinogen concentration □—□

not diverge so much as after infusion of Reptilase alone (Fig 5), and only a slight increase of the serum radioactivity was observed indicating inhibition of the secondary fibrinolytic process The immuno electrophoresis showed, however, that the fibrinolytic process was delayed, but not completely inhibited by treatment with EACA Precipitation arcs appeared in the serum samples taken 2—4 hrs after the start of the

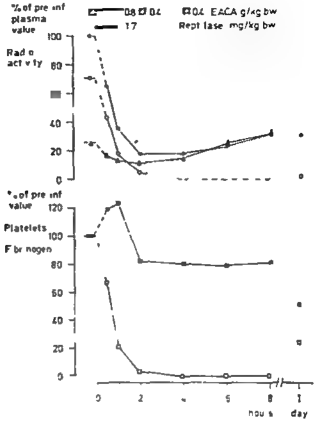

Fig 5 Simultaneous infusions of Reptilase and EACA followed by repeated injections of EACA 2 and 4 hrs later Dog No 871 Plasma radioactivity before experiment 39 0 10 ³ μC/ml
Radioactivity of plasma ●—●, fibrinogen○—○ and serum △—△
Platelets ■—■ Fibrinogen concentration □—□

EACA and Reptilase infusions (Fig 2 b and 3 b) Thus increased fibrinolytic activity occurred during EACA treatment

Group IV Trasylol and Reptilase

The fibrinogen concentration decreased during the first 6 hrs to about 15 per cent of the pre-infusion value in all 3 dogs The platelets were only moderately decreased (Table II)

After administration of Trasylol the fibrinolytic response was delayed, but not completely inhibited even if Trasylol was given in repeated doses 2 and 4 hrs after the start of the Reptilase infusion As judged from the radioactivity curves the fibrinolytic process seemed to be inhibited for 6 hrs (Fig 6) The immuno electrophoretic pattern was however similar to that obtained in EACA-treated dogs (*cf* Fig 2 b)

Group V Long-term treatment with Reptilase

The results from one of the dogs (no 871) are illustrated in Fig 7 Daily i v in tions of Reptilase kept the fibrinogen concentration on a rather constant

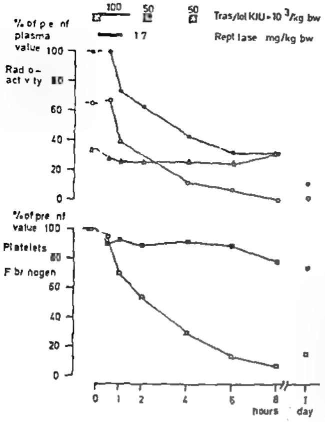

Fig 6 Simultaneous infusions of Reptilase and Trasylol followed by repeated injections of Trasylol 2 and 4 hrs later Dog No 977 Plasma 23 10 μC/ml Radioactivity of plasma ●—● fibrinogen ○—○ and serum △—△ Platelets ■—■ Fibrinogen concentration □—□

ween 0 04—0 9 g/100 ml of plasma Blood sample taken 6 hrs after one of the daily injections showed that the fibrinogen concentration decreased from 0 04 to 0 01 g/100 ml plasma An increase of the Reptilase dose did not seem to depress the fibrinogen concentration further Intramuscular injections were less effective A continuous increase of the platelet count was observed during the first 2 weeks of the treatment Immuno electrophoretic studies on serum samples during treatment showed faint precipitation lines only on the first day after the defibrinogenation as observed in the other dogs The findings in dog no 837 were in agreement with the observations in dog no 871

Discussion

Reptilase, like thrombin and thromboplastin (Nordstrom and Zetterqvist 1968 1969) is capable of inducing intravascular coagulation as shown by the disappearence of fibrinogen In terms of thrombin activity equivalents the doses required for fibrinogen depletion are much smaller for Reptilase than for thrombin This may be due to the absence of plasma inhibitors to Reptilase (Blomback *et al* 1957) For the same reason or because a slow excretion rate for Reptilase the coagulation promoting activity remains for at least 24 hrs in the blood

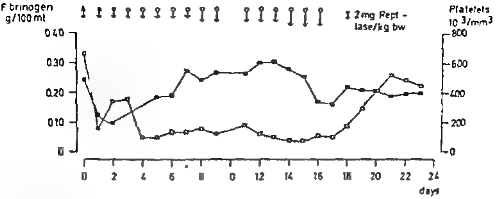

Fig 7 Long term treatment with Reptilase in Dog No 871 Platelets ■—■ Fibrinogen concentration □—□

Infusion of Reptilase △

Intramuscular injection of Reptilase ●

Intravenous injection of Reptilase ○

The Reptilase induced intravascular coagulation is followed by a release of degraded fibrin into the circulation, as indicated by the radioactivity measurements and the immunological investigations In spite of this clear evidence of degradation of fibrin no increased proteolytic (fibrinolytic) activity was demonstrated in the circulating blood Similar observations were made after thrombin infusion in dogs (Kowalski *et al* 1965, Nordstrom and Zetterqvist 1968) The fibrinolytic process may take place in the reticuloendothelial system or the vessel wall

Treatment with EACA and Trasylol makes it possible to delay and partly inhibit the secondary activated fibrinolytic process but no complete inhibition is obtained Thus, Reptilase may cause a fibrinolytic process which only in part is influenced by EACA and Trasylol This is in accordance with the findings of Reid (1965) that no thrombotic complications appeared during EACA treatment of patients bitten by the Malayan pit viper

Regoeczi, Gergely and McFarlane (1966) found that intravascular coagulation induced in rabbit by crude venom from Malayan pit vipers gave rise to a secondary hyperfibrinolysis Arvin a purified fraction of this venom was shown in clinical investigations to possess the capacity of dissolving thrombi when administered during several days (Sharp *et al* 1968 Bell Pitney and Goodwin 1968) This indicated that a persistant fibrinolysis was achieved In our experiments of long term Reptilase treatment we could not with the immunological methods used demonstrate fibrinolytic split products for more than 24 hrs after the initial defibrination The reason for this is obscure One explanation may be that the circulating amount of degradation products was too low to be detected It should also be kept in mind that only *de novo* synthetized fibrinogen was available to Reptilase coagulation and subsequent degradation Another explanation can be that Reptilase partly inhibited the syn of fibrinogen

No significantly increased bleeding tendency was observed in our experiments after defibrinogenation The main cause of the maintained hemostatis must be that the platelet function is essentially undisturbed by Reptilase Reptilase produce platelet aggregates that easily desaggregate probably because effective influnce of ADP is lacking (Morse) Jackson and Conley 1967) The slight to moderate decrease of the platelet count observed especially when larger doses of Reptilase were given may be due to traces of a thrombinplastin like activity present in the preparations used This thromboplastin like substance can cause an endogenous thrombin generation and a secondary platelet aggregation due to release of ADP This hypothesis can be supported by the fact that heparinization prevents a decrease of the platelet count, but does not abolish the defibrinogenating effect of Reptilase However EACA and Trasylol also prevent a decrease in platelet count An inhibiting effect of these compounds on brain thromboplastin has been shown (Nordstrom and Zetterqvist 1969) and an equal effect may be obtained against the thromboplastin *like substance present which might be a contaminant in the Reptilase preparations*

Reptilase decreased prothrombin at most moderately but factor V considerably The effect on factor V was studied in only one dog and will later be more thoroughly investigated

The failure to demonstrate any thrombi at autopsy in these experiments shows that a successive and complete removal of fibrin takes place As the polymerization of Reptilase fibrin is different from that of thrombin fibrin (Laurent and Blomback 1958) it is possible that a defective clot is formed A slow and defective clot formation paired with the high tendency of Reptilase fibrin to form soluble complexes opec *et al* 1968) may facilitate this removal This in combination with a rapid voked fibrinolytic process probably prevents formation of solid fibrin clots in vascular bed

Arvin was used as a therapeutic agent in patients with thromboembolic manifestations with promising results (Sharp *et al* 1968 Bell Pitney and Goodwin 1968) If the active principle of Arvin proves to be of the same nature as the thrombin like enzyme in Reptilase it is reason to believe that Reptilase like Arvin might be useful in the treatment of some thrombotic conditions

We wish to thank Dr Birger Blomback and Dr Erik Berglund for invaluable support and discussions during this work and Miss *Eivor Holmberg for most* skilful technical assistance

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References

BERGSTROM K B BLOMBACK and G KLEEN Studies on the plasma fibrinolytic activity in a case of liver cirrhosis *Acta med scand* 1960 *168* 291—305

BELL W R W R PITNEY and J F GOODWIN Therapeutic defibrination in the treatment of thrombotic desease *Lancet* 1968 *1* 490—493

BLOMBACK B Studies on the action of thrombic enzymes on bovine fibrinogen as measured by N terminal analysis *Arkiv Kemi* 1958 *12* 321—335

BLOMBACK B and M BLOMBACK Purification of human and bovine fibrinogen *Arkiv Kemi* 1956 *10* 415—443

BLOMBACK B and M BLOMBACK F rfarande for utfallning av aggviteamnen *Swedish patent application* 1966 no 10268

Blombäck B, M Blombäck and B Holmberg A new method for fractionation of proteins *Acta chem scand* 1966 *20* 2317—2319

Blombäck, B, M Blombäck and I M Nilsson, Coagulation studies on "Reptilase", an extract of the venom from Bothrops jararaca *Thrombos Diathes haemorrh* (Stuttg) 1957 *1* 76—86

Blombäck B and T C Laurent, N-terminal and light scattering studies on fibrinogen and its transformation to fibrin *Arkiv Kemi* 1958 *12* 137—146

Chan K E, *The hypofibrinogenaemic state in Malayan viper poisoning* 1964 Ph D Thesis Queen's Coll St Andrew's University

Chan K E, C R Rizza and M P Henderson, A study of the coagulant properties of Malayan pit viper venom *Brit J Haemat* 1965 *11* 646—653

E[illegible]

E[illegible]

H[illegible]

[illegible] r Slangengattung Bothrops I [illegible] 204—216

[illegible] die Gifte der Slangengattung [illegible] *nak* 1936 *181* 387—398

[illegible] L, Z Wegrzynowicz, A Z Budzynski Z S Latallo B Lipinski and E Kowalski, Interaction of fibrinogen degradation products (FDP) with platelets *Exp biol Med* 1968 *3* 73—79

Kowalski E [illegible]

K[illegible]

Laurent, T C and B Blombäck On the significance of the release of two different peptides from fibrinogen during clotting *Acta chem scand* 1958 *12* 1875—1877

McFarlane, A S, In vivo behavior of ^{131}I fibrinogen *J clin Invest* 1963 *42* 346—361

Morse, E E, D P Jackson and C L Conley Effects of reptilase and thrombin on human blood platelets and observations on the molecular mechanism of clot retraction *J Lab clin Med* 1967 *70* 106—115

Nordstrom, S and B Zetterqvist, Effects of thrombin infusions upon ^{131}I labelled fibrinogen in dogs *Acta physiol scand* 1968 *72* 85—99

Nordstrom, S and E Zetterqvist, Effects of epsilon aminocaproic acid (EACA) and Trasylol on thromboplastin-induced intravascular coagulation studied in dogs with iodine labelled fibrinogen *Acta physiol scand* 1969 *76* 93—105

Noren I and M Blombäck New rapid tests for coagulation disorders *Scand J clin Lab Invest* 1965 *17* Suppl 86

Nussenzweig, V, M Seligmann and P Grabar Les produits de degradation du fibrinogene humain par la plasmine II Etude immunologique Mise en evidence d'anticorps anti fibrinogene natif possidant des specificites differentes *Ann Inst Pasteur* 1961 *100* 490—508

Ouchterlony, Ö, Antigen-antibody reactions in gels Factors determining the site of the precipitate *Arkiv Kemi* 1949 *1* 43—59

Regoeczi, E, J Gergely and A S McFarlane In vivo effects of Agkistrodon rhodostoma venom Studies with Fibrinogen ^{131}I *J clin Invest* 1966 *45* 1202—1212

Reid H A, E aminocaproic acid and fibrinolysis in viper-bite defibrination *Lancet* 1965 *2* 5—7

Reid H A, *Defibrination by Agkistrodon rhodostoma venom* First int Symp Animal Toxins (1966) Pergamon Press Oxford and New York 1967 323—335

Scheidegger, J J Une micro methode de l'immuno-electrophorese *Int Arch Allergy* 1955 *7* 103—110

Sharp A A, B A Warren A M Paxton and M J Allington Anticoagulant therapy with a purified fraction of Malayan pit viper venom *Lancet* 1968 *1* 493—499

Steiner, R F and K Laki Light scattering studies on the clotting of fibrinogen. *Arch Biochem* 1951 *34* 24—37

Wolf P A, A modification for routine laboratory use of Stefanini's method of estimating factor V activity in human oxalated plasma *J clin Path* 1953 *6* 34—38

Zetterqvist, E Fibrinogen degradation products in experimental intravascular coagul[illegible] Gel filtration and immunological studies *Acta physiol scand* 1970 In press

Acta physiol. scand. 1970. 79. 506—515

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Estimation of Ionic Concentrations and Intracellular pH in Slices from Different Areas of Rat Brain

By

L. Hertz, A. Schousboe and George B. Weiss[1]

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Abstract

Hertz, L., A. Schousboe and G. B. Weiss. *Estimation of ionic concentrations and intracellular pH in slices from different areas of rat brain.* Acta physiol. scand. 1970. 79. 506—515.

Tissue spaces occupied by ^{14}C-sucrose and ^{3}H-inulin were measured at different incubation time intervals (15—120 min) in rat brain slices prepared from cortex, cerebellum, caudate, diencephalon and midbrain. Total water content, total Na^+ and K^+ contents and ^{14}C-DMO (5,5-dimethyl-2,4-oxazolidinedione) spaces were obtained from the same tissues. At all time intervals ^{14}C-sucrose spaces were larger than corresponding ^{3}H-inulin spaces. After 60 min incubation, the mean values for ^{14}C-sucrose spaces in preparations from different brain areas ranged from 31.5 to 38.1 ml/100 g, whereas the ^{3}H-inulin space mean values were 19.3—26.1 ml/100 g. Average intracellular pH was calculated from ^{14}C-DMO spaces in the presence and absence of 3.67 mM procaine. The pH_i values obtained using inulin derived extracellular spaces were 6.91—6.99, and those calculated from sucrose spaces were 6.77—6.89. The contents of K^+ and Na^+ showed no distinct regional differences, but a potassium uptake with time could only be observed in preparations from cerebral and cerebellar cortex.

Characterization of the extracellular space in brain tissue has been the objective of numerous studies both *in vivo* and *in vitro* (e.g. Pappius and Elliott 1956, Horstmann and Meves 1959, Van Harreveld 1966). However, both the magnitude of the extracellular space and the homogeneity of the intracellular space remain uncertain even in a well-defined preparation as the brain cortex (Schousboe and Hertz 1970), and information about possible regional differences in water metabolism is scarce (cf. Aprison, Lukenbill and Segar 1960).

More consistent information is found when ionic contents are considered. The potassium concentration in fresh grey matter is about 100 μmoles/g and the sodium concentration is about half of this value (Yannet 1940, Ames and Nesbett 1958, Bachelard, Campbell and McIlwain 1962), the differences between different regions (Aprison *et al.* 1960) and even between white and grey matter are relatively small

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(Stewart Wallace 1939, Levy *et al* 1965, Tower 1969) Under optimal and physiological *in vitro* conditions the potassium concentration of brain cortex slices has been found to be about 70—75 mmoles/g final wet wt (Franck Cornette and Schoffeniels 1968 Lund Andersen and Hertz 1970) Again, virtually no information is available about tissues from other brain regions

A few measurements of intracellular pH in brain cortex *in vivo* have given values around 7 0 (Brodie and Woodbury 1958 Koch and Woodbury 1960) but no in vitro studies appear to have been performed

The present study represents an attempt to obtain comparative values for tissue compartment sizes and for cationic contents and distribution at different incubation time intervals in slices prepared from different areas of the rat brain The purpose was to obtain incubation time intervals which would prove suitable for further comparative studies concerning equilibration and movement of different types of molecules in the brain area slices Previous studies of ion movements in rat cortex slices (Hertz 1968) and of ion and drug movements in isolated muscle (Weiss 1966, 1968a, 1969) have yielded information under similar circumstances Since procaine alters the [14]C-DMO space and resultant pH, in frog sartorius muscle (Bianchi and Bolton 1967, Weiss 1968b) a comparison of the pH, values obtained in the presence and absence of procaine was also undertaken

Methods

Brain sections were obtained from Wistar rats weighing 200—250 g All animals were decapitated while under light ether anesthesia, brains were rapidly removed and brain areas were prepare

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agitation of the slices (Hertz et al 1970) The

he incubation period

s slices were placed in tubes containing 4 ml of

uniformly labeled [14]C sucrose (specific activity

g) After the desired incubation interval slices were removed from the radioactive

When inulin spaces were also determined, 20 0 μC of ^{3}H-methoxyinulin (molecular weight 5000—5500, specific activity 0 1 mC/mg) was added to 4 ml of the incubation solution containing ^{14}C-sucrose After extraction of the slices, ^{3}H methoxyinulin and ^{14}C sucrose tissue spaces were obtained by counting the radioactivity of each compound using appropriately adjusted channels of the spectrometer and suitable correction factors

The tissue space values have in this study been expressed in terms of ml per 100 g of the final wet weight of the tissue, *i e* in per cent of the final wet weight This is obtained by dividing the total amount of radioactive compound taken up per 100 g tissue by the concentration of radioactive compound per ml of the incubation solution It should be noted that this expression is at variance with that generally used in neurochemical work (*e g* Pappius and Elliott 1956 Bourke and Tower 1966, Schousboe and Hertz 1970) in which spaces are expressed per unit initial wet weight

Total Na^+ and K^+ contents were measured by means of a Unicam SP 90 flame photometer The values were corrected for the interference by Na^+ with K^+ readings (Arnfred *et al* 1970) Total Na^+ and K^+ contents were estimated on the basis of both the final wet weights and the dry weights

The intracellular pH (pH_i) was calculated from the ^{14}C DMO (5,5 dimethyl-2,4-oxazolidinedione) space (Waddell and Butler 1959, Bianchi and Bolton 1967) using the following equation

$$pH_i = 6\,13 + \log ((DMO/100(1 + V_e/V_i) - V_e/V_i)(10^{pH_e - 6} + 1) - 1)$$

... IO at 37° (Bittar 1964), DMO the DMO space ... intracellular water space (100—the amount of ... DMO spaces were obtained by incubating slices ... ^{14}C DMO (specific activity 62 μC/mg) After ... were placed overnight in distilled water at 4° Aliquots from the extracts and from dilutions of the ^{14}C DMO incubation solutions were counted in the same manner as the ^{14}C-sucrose samples

The non-radioactive chemicals were purest grade Procaine was added as the hydrochloride to a concentration of 3 67 mM in the incubation medium The radioactive compounds were obtained from New England Nuclear Corp, Boston, Mass

Results

Estimation of inulin and sucrose spaces

Measurements of the 'extracellular' tissue compartment in different rat brain areas was attempted with both ^{14}C-sucrose and ^{3}H-inulin Tissue slices were extracted after different incubation time intervals and the resultant time course of uptake of radioactive compound into cortex slices is shown in Fig 1 The uptake of both substances is initially relatively rapid and then reaches a maximum The ^{14}C-sucrose space markedly exceeds the ^{3}H-inulin space

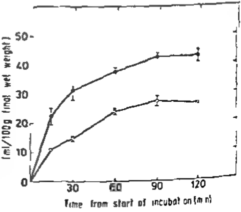

Fig 1 Uptake of ^{14}C-sucrose (● and of ^{3}H-inulin (○) into rat brain-cortex slices as a function of the time of incubation The uptakes are represented as 'spaces' *i e* the fractions of the tissue (expressed as percentages) which contain the same concentrations of the radioactive compounds as the incubation media

Results are means of 2—6 expts vertical bars indicate standard error under conditions where the average for at least 3 expts is given

TABLE I ^{14}C-sucrose and ^{3}H inulin tissue spaces in slices obtained from different rat brain areas*

^{14}C-sucrose space in	Duration of incubation time (min) 15	30	60	90	120
			ml/100g ± S.E.M.		
Cortex	22.4 ± 3.2 (4)	31.5 ± 3.4 (6)	37.8 ± 1.4 (6)	42.7 ± 1.3 (6)	42.8 ± 2.2 (4)
Cerebellum	23.8 ± 3.4 (4)	22.0 ± 1.1 (5)	31.5 ± 3.0 (6)	32.3 ± 1.4 (5)	42.5 ± 1.0 (4)
Caudate	26.1 ± 3.3 (3)	33.9 ± 3.8 (6)	34.0 ± 2.4 (6)	40.0 ± 2.3 (6)	47.8 ± 2.6 (4)
Diencephalon	23.7 ± 1.4 (3)	24.7 ± 2.2 (5)	32.0 ± 1.6 (6)	36.8 ± 3.8 (6)	41.4 ± 4.1 (3)
Midbrain	24.3 ± 3.3 (3)	31.8 ± 1.2 (5)	38.1 ± 3.1 (6)	38.8 ± 1.7 (5)	48.0 ± 3.1 (3)
^{3}H inulin space in					
Cortex	11.1 (2)	14.7 ± 0.6 (3)	24.0 ± 0.9 (4)	27.4 ± 1.9 (3)	26.5 (2)
Cerebellum	13.5 (2)	13.6 (2)	19.3 ± 3.4 (4)	24.6 ± 2.0 (3)	29.9 (2)
Caudate	17.7 (2)	17.2 ± 2.0 (3)	25.1 ± 1.1 (4)	32.1 ± 2.7 (3)	31.0 (2)
Diencephalon	14.8 (2)	18.2 ± 1.2 (3)	20.1 ± 0.5 (4)	25.1 ± 3.1 (3)	30.5 (2)
Midbrain	17.5 (2)	18.0 (2)	26.1 ± 3.0 (4)	29.3 ± 2.7 (3)	36.3 (2)

* Values represent the mean ± S.E.M. for all conditions where N = 3 or more. The mean is given where only two values were obtained. N is given in parentheses after each value. All values are expressed as ml/100 g final wet weight.

Also in cerebelleum, caudate, diencephalon and midbrain the ^{14}C-sucrose spaces are significantly greater than corresponding ^{3}H-inulin spaces (Table I). As was illustrated in Fig. 1 for cortex slices, all tissue preparations initially gain ^{14}C-sucrose or ^{3}H-inulin at a rapid rate. However, in a number of instances the tissue spaces continue to increase between 90 and 120 min incubation time. The tissue space values obtained at similar incubation time intervals and with the same isotope do not differ from one brain area to another.

Measurement of tissue water space

The percentages of tissue water ((final wet weight minus dry weight/final wet weight) × 100) are summarized in a similar manner in Table II. The water content increases as the duration of incubation is lengthened. This increase, which indicates

TABLE II Percentage tissue water content in slices obtained from different rat brain areas*

Brain area	Duration of incubation time (min) 15	30	60	90	120
Cortex	83.3 ± 0.2 (4)	83.8 ± 0.1 (6)	84.5 ± 0.1 (6)	84.6 ± 0.2 (6)	85.0 ± 0.1 (4)
Cerebellum	82.6 ± 0.5 (4)	83.2 ± 0.1 (5)	84.4 ± 0.1 (6)	85.3 ± 0.4 (5)	85.2 ± 0.3 (4)
Caudate	81.6 ± 0.7 (3)	82.4 ± 0.7 (6)	83.8 ± 0.3 (6)	84.4 ± 0.4 (6)	86.1 ± 0.3 (4)
Diencephalon	81.1 ± 0.6 (3)	82.2 ± 0.4 (5)	81.5 ± 0.5 (6)	83.1 ± 0.6 (6)	82.9 ± 0.5 (3)
Midbrain	77.8 ± 1.6 (3)	80.0 ± 1.2 (5)	81.2 ± 0.7 (6)	81.7 ± 0.7 (5)	81.6 ± 0.9 (4)

* Values represent the mean ± S.E.M. N is given in parentheses after each value. All values were obtained by dividing the difference between the final wet and dry weights by the final wet weight and are expressed as percentages. Tissues analyzed were those employed for the sucrose spaces listed in Table I.

TABLE III Total Na^+ contents in slices from different rat brain areas*

Na^+ in	Duration of incubation time (min)				
	15	30	60	90	120
Cortex	97.0 ± 7.5 (580 ± 51)	87.3 ± 3.3 (546 ± 22)	83.7 ± 3.6 (541 ± 28)	82.6 ± 4.4 (536 ± 22)	86.2 ± 6.[illegible] (575 ± 40
Cerebellum	97.4 ± 4.6 (577 ± 14)	100.8 ± 8.[illegible] (601 ± 51)	92.3 ± 4.2 (593 ± 30)	100.3 ± 10.5 (687 ± 85)	104.3 ± 7.[illegible] (702 ± 46
Caudate	94.7 ± 3.3 (547 ± 36)	102.0 ± 6.[illegible] (586 ± 43)	92.1 ± 10.3 (566 ± 72)	103.0 ± 2.5 (693 ± 26)	112.6 ± 6.[illegible] (812 ± 52
Diencephalon	89.1 ± 4.7 (474 ± 41)	88.6 ± 4.5 (498 ± 23)	94.4 ± 5.1 (512 ± 32)	98.0 ± 9.3 (584 ± 60)	86.4 ± 3.[illegible] (506 ± 5)
Midbrain	95.9 ± 6.8 (435 ± 37)	106.2 ± 7.3 (537 ± 45)	104.2 ± 5.8 (558 ± 38)	103.6 ± 5.5 (578 ± 48)	97.1 ± 4.[illegible] (532 ± 40

* Values represent μmoles Na^+ per g final wet weight ± S.E.M. The equivalent values calcu- lated for μmoles Na^+ per g dry weight ± S.E.M. are given in parentheses. The tissues em- ployed were identical in all cases to those listed in Table II.

that a gradual swelling of the tissue takes place, is especially marked in the caudat and cerebellum slices but also present in slices from the other regions. The midbrai and diencephalon slices have lower water spaces than the other three brain area slice. These preparations are not only more heterogenous, but contain much larger amount of white matter.

Sodium and potassium ion contents and concentrations

The total amounts of Na^+ and K^+ in the tissue have been expressed both per unit fina weight and per unit dry weight. The sodium content (*i.e.* μmoles/g dry wt.) ir cortex slices is almost unaltered during the length of the incubation (Table III). In all other tissues it rises with time and the augmentation is most pronounced anc continuous with tissue from the caudate and from the cerebellum. The concomitant

TABLE IV Total K^+ contents in slices from different rat brain areas*

K^+ in	Duration of incubation time (min)				
	15	30	60	90	120
Cortex	57.8 ± 2.3 (346 ± 18)	59.4 ± 3.7 (368 ± 24)	59.5 ± 1.6 (385 ± 9)	63.8 ± 2.2 (415 ± 12)	64.6 ± 4.2 (430 ± 25)
Cerebellum	56.7 ± 8.8 (326 ± 51)	54.7 ± 3.9 (326 ± 23)	59.0 ± 5.0 (379 ± 34)	58.9 ± 2.5 (401 ± 22)	55.5 ± 4.2 (374 ± 25)
Caudate	50.6 ± 2.3 (293 ± 25)	49.6 ± 3.0 (290 ± 20)	42.4 ± 2.4 (263 ± 19)	49.3 ± 2.8 (330 ± 14)	42.7 ± 6.2 (309 ± 49)
Diencephalon	59.4 ± 1.3 (315 ± 9)	52.9 ± 3.7 (303 ± 20)	51.0 ± 3.9 (277 ± 24)	56.8 ± 2.6 (355 ± 24)	51.3 ± 5.8 (303 ± 43)
Midbrain	50.7 ± 2.3 (232 ± 27)	47.9 ± 2.5 (244 ± 25)	51.9 ± 4.3 (278 ± 18)	43.1 ± 5.4 (327 ± 77)	44.7 ± 3.9 (243 ± 18)

* Values represent μmoles K^+ per g final wet weight ± S.E.M. The equivalent values calcu- lated for μmoles K^+ per g dry weight ± S.E.M. are given in parentheses. The tissues em- ployed were identical in all cases to those listed in Table II.

TABLE V Intracellular pH values calculated from ^{14}C-DMO space measurements in paired rat brain slices in the absence and in the presence of procaine*

Experimental Conditions	^{14}C-DMO Space (ml/100g ± S.E.M.)	pH_i using ^{3}H inulin space	pH_i using ^{14}C-sucrose space	^{14}C-DMO Space Difference (ml/100g ± S.E.M.)	P**
Cortex					
Control	70.9 ± 0.5	6.95	6.83	2.9 ± 1.0	< .01
Procaine	73.8 ± 0.8	6.98	6.88		
Cerebellum					
Control	67.7 ± 1.1	6.94	6.85	5.4 ± 1.4	< .005
Procaine	73.1 ± 0.8	6.99	6.93		
Caudate					
Control	68.2 ± 1.3	6.91	6.87	1.8 ± 1.7	< .25
Procaine	70.0 ± 1.0	6.93	6.85		
Diencephalon					
Control	70.9 ± 1.3	6.96	6.87	0.5 ± 1.7	< .40
Procaine	71.4 ± 1.3	6.97	6.89		
Midbrain					
Control	69.3 ± 1.3	6.91	6.77	1.2 ± 2.2	< .40
Procaine	70.5 ± 2.0	6.92	6.80		

* The time of exposure to ^{14}C-DMO was 60 min. Procaine was also present for the entire 60 min period. Data from 60-min values in preceding Tables were used for calculations. All ^{14}C-DMO values represent the average for 8 pairs except those for midbrain, which include 7 pairs.

** The level of significance (P) is calculated for the average of the differences between paired slices using students t test.

changes in sodium concentration (*i.e.* µmoles/g final wet wt.) are much less marked indicating that the sodium uptake with time quantitatively almost corresponds to the swelling.

The potassium content (Table IV) in cortex slices increases steadily during the hrs of incubation ($P<0.05$); *similar tendency is observed in cerebellar tissue,* whereas the potassium content in the caudate, the diencephalon and the midbrain is essentially the same after 120 min of incubation as after 15—30 min of incubation. As was the case with the uptake of sodium (Table III) the potassium concentration in the cerebral and cerebellar slices does not increase correspondingly, again indicating a relation between uptake of an ion and of water. In the caudate, the diencephalon and the midbrain the potassium concentration tends to decline with time.

Intracellular pH

Average intracellular pH values (pH_i) were calculated from DMO spaces in conjunction with extracellular (*i.e.* inulin or sucrose) spaces and water spaces (Table V). The pH_i values calculated from the ^{3}H inulin spaces are only slightly higher than those derived from the ^{14}C sucrose spaces and approximately identical in the 5 regions. Procaine increases the ^{14}C DMO space slightly in cortical and cerebellar slices.

Discussion

The present study demonstrates that tissue spaces obtained with ^3H inulin are lower than corresponding ^{14}C sucrose spaces in slices prepared from all brain areas studied. The question of whether this difference between sucrose and inulin spaces represents an incomplete filling of the extracellular tissue space by inulin or an uptake of some sucrose into the intracellular compartment is one which has in the past been a problem in different tissues. In smooth muscle a number of substances which might measure extracellular space were found to give different values for this tissue compartment (Goodford and Hermansen 1961, Barr and Malvin 1965). Explanations for this variation in extracellular space estimates have included a hypothesis that multiple water compartments exist (Barr and Malvin 1965), a finding that steric hindrance prevents inulin entry into a portion of the extracellular compartment (Goodford and Leach 1966) and a report that sucrose enters the smooth muscle cell (Bozler and Lavine 1958). However, if a thin tissue (such as the longitudinal smooth muscle layer of the guinea pig ileum) is used, diffusion is not a problem and sucrose and inulin spaces are similar (Weiss 1966). Whether hindrance of inulin movement may be of importance in slices from some brain areas is not resolved by this study. In slices from the brain cortex evidence indicates, however, that both sucrose and inulin have access to at least one intracellular compartment (Bourke and Tower 1966, Cohen *et al.* 1968, Cohen and Lajtha 1969, Schousboe and Hertz 1970), and that both neurons and glial cells are permeable to these markers (Nicholls and Wolfe 1967, Brown, Stumpf and Roth 1969). In view of the differences in histological structure between cerebral cortex, cerebellar cortex, caudate, diencephalon and midbrain, it is remarkable that the difference between the two spaces is essentially the same in the different regions (10—15 % of the final wet weight of the tissue) and almost independent of the length of the incubation period.

Determination of extracellular space is a prerequisite for determination of intracellular pH, and ^{14}C DMO spaces were employed along with either sucrose or inulin spaces to derive pH values. The selection of either sucrose or inulin space for the calculation did not greatly alter the pH values obtained. The probable compartmentation of the intracellular phase (*e.g.* Schousboe and Hertz 1970) means, however, that the pH must be regarded only as an average value (*cf.* Roos 1965). The small effect evoked by addition of procaine to the incubation solution contrasts with the marked increase in ^{14}C DMO space observed in frog sartorius muscles when the same concentration of procaine (3.67 mM) was employed (Weiss 1968b).

In a similar way, average concentrations of Na and K in the intracellular water phase might conceivably be calculated on the basis of either inulin or sucrose spaces. Table VI shows the concentrations derived from 60 min values for total water content, for total Na and K concentration and for either ^{14}C sucrose or ^3H inulin space. The estimates of the intracellular concentrations depend strongly upon the space used for determination of the extracellular water phase and in most cases the joint intracellular concentrations of Na and K greatly exceed the cationic con-

TABLE VI Calculation of average Na⁺ and K⁺ concentrations (µmoles/g final wet weight) in the tissue compartments which are nonpermeable to either inulin or sucrose *

Brain Area Slice	Calculated from ^{3}H inulin space		Calculated from ^{14}C-sucrose space	
	Na^+	K^+	Na^+	K^+
Cortex	84 8	96 4	69 8	123 3
Cerebellum	101 8	89 1	94 1	108 5
Caudate	99 1	70 0	92 8	81 7
Diencephalon	109 4	81 4	103 4	99 8
Midbrain	125 2	91 8	122 5	116 0

* Values calculated from 60 min incubation period averages for ^{3}H inulin space ^{14}C sucrose space (ml/100 g final wet wt), contents of solids (g/100 g final wet wt) and Na^+ and K^+ concentrations (µmoles/g final wet wt) as listed in Table I II, III and IV

For the calculations the following equation was used

ion concentration = (a—x b/100) 100 (100—(b +c)

in which x) is the bathing medium concentration (i e 135 mM Na^+ or 5 mM K^+ a the tissue concentration of either Na^+ or K^+, b) the magnitude of either inulin of sucrose space, and c) the contents of solids

centration in the medium *i e* about 150 mM The latter finding indicates that in brain tissue calculation of intracellular concentrations on the basis of either inulin or sucrose space on the assumption that the ionic concentration in these spaces are similar to those in the incubation medium does not give any estimate of true intra cellular concentrations Conceivably one source of error might be that significant amount of Na^+ or K^+ were bound to non aqueous cellular material as seems to be the case in smooth muscle (von Hagen and Hurwitz 1967) where only fractions of the cellular Na^+ and K^+ participate in the maintenance of functional ion gradients across the cell membrane (Weiss 1969) No such binding occurs in cortical tissue (H Hillman personal communication, *cf* also the potassium concentrations under anoxia reported by Lund Andersen and Hertz 1970) but it might occur in tissues containing a greater proportion of white substance (*cf* the very high intracellular' concentrations of Na^+ and of K^+ in midbrain in Table VI) On the basis of these considera tions the calculations of cellular distribution of Na^+ and K^+ must be regarded as un certain in all cases (*cf* Schousboe and Hertz 1970) but even more so in slices from brain regions other than cortex

Both the intracellular concentrations and the concentrations of Na^+ and K^+ expressed in relation to the total amount of tissue (*i e* per unit final wet weight) are remarkably similar in the 5 different regions This is analogous to the resemblance found *in vivo* (Aprison *et al* 1960, Hanig and Aprison 1967), but the K^+ concentrations are considerably lower and the Na^+ concentrations higher The relative constancy of the ionic concentrations with time seems to be correlated with changes in water content An increased water uptake evoked by accumulation of potassium and chloride ions without concomitant extrusion of sodium ions has previously been described in slices from brain cortex (Bourke 1969, Lund-Andersen and Hertz 1970)

The increase in potassium content (i e, K^+ per unit dry weight) as a function of time in cerebellar cortex (Table IV) probably indicates the presence of a similar mechanism in this area The lack of potassium uptake in slices from the other 3 regions might conceivably be due either to more severe destruction of these slices during preparation or to the absence of a mechanism for uptake of potassium and chloride ions

More definite answers to the problems of such regional differences within the brain probably require studies in species with larger brains where well defined uncontaminated tissue can be more readily obtained from specific regions The major purpose of this study was, however, to obtain values for such cellular parameters as 'extracellular' space, water space, ionic contents and pH gradients in laboratory animals in which pharmacological and behavioural studies may also be performed with relative ease These values are useful in describing the equilibration and movement of molecules or ions in different brain areas Nicotine distribution in muscle appears to be in agreement with a calculated distribution based on the pH gradient (Weiss 1968a) The data obtained in the present study may be of value in making similar calculations using brain slices for this and other compounds of interest with pK_a values in the physiological pH range Similarly, it could facilitate investigation of possible correlations between the actions of substances on the distribution or release of radioactive ions or molecules in different brain areas and the presumed site of action of these same substances *in vivo*

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References

AMES A and F B NESBETT A method for multiple electrolyte analyses on small samples of nervous tissue *J Neurochem* 1958 *3* 116—126

APRISON M H A LUKENBILL and W E SEGAR, Sodium, potassium, chloride and water content of six discrete parts of the mammalian brain. *J Neurochem* 1960 *5* 150—155

ARNFRED T L HERTZ L LOLLE and H LUND-ANDERSEN, Intense oxygenation and free mobility as a method to increase the potassium content in transferred and non transferred brain slices In preparation 1970

BACHELARD H S W J CAMPBELL and H MCILWAIN The sodium and other ions of mammalian cerebral tissues maintained and electrically stimulated in vitro *Biochem J* 1962 *84* 225—232

BARR, L and R L MALVIN Estimation of extracellular spaces of smooth muscle using different sized molecules *Amer J Physiol* 1965 *208* 1042—1045

BIANCHI C P and T C BOLTON Action of local anesthetics on coupling systems in muscle *J Pharmacol exp Ther* 1967 *157* 388—405

BITTAR E E, *Cell pH* Butterworths London. 1964

BOURKE R. S Evidence for mediated transport of chloride in cat cerebral cortex in vitro *Exp Brain Res* 1969 *8* 219—231

BOURKE, R. S and D B TOWER Fluid compartmentation and electrolytes of cat cerebral cortex in vitro — I Swelling and solute distribution in mature cerebral cortex *J Neurochem* 1966 *13* 1071—1097

BOZLER E and D LAVINE Permeability of smooth muscle *Amer J Physiol* 1958 *195* 45—49

BRODIE, D A. and D M WOODBURY Acid base changes in brain and blood of rats exposed to high concentrations of carbon dioxide *Amer J Physiol* 1958 *192* 91—94

BROWN, D. A. W. E. STUMPF and L. J. ROTH. Location of radioactively labelled extracellular fluid indicators in nervous tissue by autoradiography. *J. cell Sci.* 1969, *4*, 265—288.
COHEN, S. R., R. BLASBERG, G. LEVI and A. LAJTHA. Compartmentation of the inulin space in mouse brain slices. *J. Neurochem.* 1968, *15*, 707—720.
[illegible]
GOODFORD, P. J. and K. HERMANSEN. Sodium and potassium movements in the unstriated muscle of the guinea pig taenia coli. *J. Physiol.* (Lond.) 1961, *158*, 426—448.
GOODFORD, P. J. and E. H. LEACH. The extracellular space of the smooth muscle of the guinea pig taenia coli. *J. Physiol.* (Lond.) 1966, *186*, 1—10.
HANIG, R. C. and M. H. APRISON. Determination of calcium, copper, iron, magnesium, manganese [illegible]
[illegible]
ton. *Amer. J. Physiol.* 1960, *198*, 434—440.
LEVY, W. A., I. HERZOG, K. SUZUKI, R. KATZMAN and L. SCHEINBERG. Method for combined ultrastructural and biochemical analysis of neural tissue. *J. Cell Biol.* 1965, *27*, 119—132.
[illegible]
1514—1532.
PAPPIUS, H. M. and K. A. C. ELLIOTT. Water distribution in incubated slices of brain and other tissues. *Canad. J. Biochem.* 1956, *34*, 1007—1022.
ROOS, A., Intracellular pH and intracellular buffering power of the cat brain. *Amer. J. Physiol.* 1965, *209*, 1233—1246.
SCHOUSBOE, A. and L. HERTZ. Effects of potassium on indicator spaces and fluxes in brain cortex slices from adult and new born rats. Submitted to *J. Neurochem.* 1970.
STEWART-WALLACE, A. M. A biochemical study of cerebral tissue and of the changes in cerebral oedema. *Brain* 1939, *62*, 426—438.
TOWER, D. B., *Handbook of Neurochemistry* (ed. A. Lajtha). Plenum Press, New York 1969, 1—24.
VAN HARREVELD, A. *Brain tissue electrolytes. Molecular biology and medicine series* (ed. E. E. Bittar), Butterworths, London 1966, 1—171.
VON HAGEN, S. and L. HURWITZ. Effect of extracellular Na and Ca ions on K movements in smooth muscle. *Amer. J. Physiol.* 1967, *213*, 579—586.
WADDELL, W. J. and T. C. BUTLER. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *J. clin. Invest.* 1959, *38*, 720—729.
WEISS, G. B., Homogeneity of extracellular space measurements in smooth muscle. *Amer. J. Physiol.* 1966, *210*, 771—776.
WEISS, G. B. Dependence of nicotine-C^{14} distribution and movements upon pH in frog sartorius muscle. *J. Pharmacol. exp. Ther.* 1968a, *160*, 135—147.
WEISS, G. B. Actions of procaine on nicotine-induced effects in frog sartorius muscle. *J. Pharmacol. exp. Ther.* 1968b, *160*, 148—158.
WEISS, G. B. Alterations of ^{22}Na distribution in ileal smooth muscle. *Amer. J. Physiol.* 1969, *217*, 878—834.
YANNET, H. Changes in the brain resulting from depletion of extracellular electrolytes. *Amer. J. Physiol.* 1940, *128*, 683—689.

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Chylomicron Size and its Relation to the Composition of Dietary Fat in the Rat

By

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Abstract

SYLVÉN, C *Chylomicron size and its relation to the composition of dietary fat in the rat* Acta physiol scand 1970 79 516—522

[illegible] ved in medium [illegible] Lymph was [illegible] The amount of lymph lipoproteins was estimated by their lipid content Gel chromatography showed that there was a continuous distribution of lymph lipoproteins according to molecular size The transport of chylomicron lipoproteins i e those of $S_f > 400$ increased with fatty acid chain length of the triglyceride used When MCT or HCOCO were administered the transport of lipoproteins $400 > S_f > 20$ increased Administration of triolein gave the same transport as HCOCO While the transport of both lipoprotein classes was lowest when MCT was used this triglyceride induced the highest transport of cholesterol in lipoproteins $400 > S_f > 20$ This was reflected in the percentage composition of the lipoprotein lipid It contained more cholesterol and less triglyceride as compared to the other dietary conditions Exogenous cholesterol was transported in both lipoprotein fractions

Exogenous lipids are in the chyle transported in lipoproteins The main part of these are chylomicrons i e they have a S_f-value of >400 (Cornwell and Kruger 1961) A substantial part however of the lipids are transported in lipoproteins $400 > S_f > 20$ Within and between the two lipoprotein classes there is a continuous distribution of molecules so that their size vary and accordingly also their density (Freeman, Lindgren and Nichols 1963 Their metabolism is dependent on this variation (Schotz, Arnesjo and Olivecrona 1966 Qwarfordt and Goodman 1965)

Zilversmit Sisco and Yokoyama (1966) have shown that the size distribution of chylomicrons is the same whether cream or corn oil is fed However, when triglyceride of different fatty acid chain length is fed, absorbed triglyceride split products of medium fatty acid chain length are not transported from the intestine via the lymph but via the portal blood When cholesterol is fed in such a medium chain triglyceride practically no exogenous triglyceride is transported via the lymph while exogenous cholesterol is transported by this route (Sylvén and Borgström 1969)

This situation might result in an increased lymphatic transport of lipoproteins smaller in size and with higher density.

In this investigation, determinations have been made on the composition of thoracic duct lymph lipoproteins after administration of cholesterol dissolved in triglyceride of different fatty acid chain length. Fractionation of lipoproteins has been made by ultracentrifugation or by gel chromatography.

Abbreviations

Hydrogenated Coconut Oil: HCOCO
Medium Chain Triglyceride: MCT

Materials

Cholesterol 7-^{3}H and cholesterol 4-^{14}C were purchased from The Radiochemical Centre, Amersham, Bucks, England. They were found by TLC to have a radiopurity of more than 97 per cent.

Medium chain triglyceride (MCT), mean chain length of the fatty acids 8.4 C, was obtained from Drew Chem. Corp., New York, U.S. and hydrogenated coconut oil (HCOCO), mean fatty ... l Refinery, Sweden ... England

... n Bio-Rad Laboratories, Richmond, California, U.S.

Methods

Experiments with thoracic duct cannulated rats were performed as previously reported (Sylven and Borgström 1969). The lymph was sampled for 8 hours after administration of the test sol ... remove the cells. Fractionation was

... as a supranatant consisting approxima... lipoproteins of S_f>400 (... et al. 1966) and a nearly homogenous infranatant consisting mainly of lipoproteins S_f<400. These two lipoprotein fractions will be referred to as S_f>400 and S_f<400.

After ultracentrifugation, the bottom of the tube was punctured and about two thirds of the infranatant withdrawn for analysis. The composition of the flotated chylomicrons were calculated from the difference between the original lymph and the infranatant.

Organic phosphorus (Chen, Toribara and Warner 1956), esterbonds (Snyder and Stephens 1959), cholesterol (Abell et al. 1952) and radioactivity (Sylven and Borgström 1969) were determined. Total lipid was calculated on the basis of the different estimations.

The test solution consisted of 50 µmoles radioactive cholesterol (^{3}H or ^{14}C) dissolved in 0.8 ml MCT, HCOCO or triolein. In experiments on fasting animals 1.0 ml of a micellar solution was administered. It was composed of a radioactive cholesterol tracer in 10 mM monoolein, 10 mM NaTDC and 150 mM NaCl (Hofmann 1963). Each test solution was administered to four rats and each rat used for two tests. The first day a test solution containing cholesterol ^{3}H was administered while the second day one with cholesterol ^{14}C was given.

... et al. (1968) 10 ml ... min. Elution was per... rom the eluted frac... 1 : 10 or 1 : 20. The ... Successive fractions were pooled in groups of two. An aliquot was taken and total lipids extracted accord... the Folch procedure. Determinations on the different lipid classes were made as ... ultracentrifugation. The test solutions consisted of 50 µmoles radioactive cho... ^{14}C) dissolved in 0.8 ml triolein or MCT. Two tests were performed for each sol...

Results

Ultracentrifugal analysis

Administration of cholesterol in triglyceride resulted in a transport of total lipid in lymph lipoprotein $S_f>400$ and $S_f<400$ as shown in Fig 1 When the carrier triglyceride was MCT there was as compared to the fasting state a minor increase in the total lipid transport of lipoproteins $S_f>400$ Further increase in mean fatty acid chain length of the cholesterol carrier resulted in a marked increase in lipid transport in this form In lipoprotein $S_f<400$ the total lipid transport increased with fatty acid chain length until HCOCO was used Triolein as carrier triglyceride gave no further increase The fraction of total lipid transported in the thoracic duct lymph in lipoproteins $S_f<400$ was for MCT as carrier triglyceride found to be 50 % while there was a decrease for triolein to 20 %

Fig 2 gives μmoles cholesterol transported in the two lipoprotein fractions The amount carried by lymph lipoproteins $S_f<400$ increased two fold as compared to the fasting state after administration of cholesterol dissolved in MCT (Fig 2) With increasing fatty acid chain length of the carrier triglyceride the transport of cholesterol in this lipoprotein fraction decreased so that it was equal to the fasting state when triolein was used The amount of cholesterol transported in lipoproteins $S_f>400$ increased with chain length Exogenous cholesterol was transported in both lipoprotein fractions

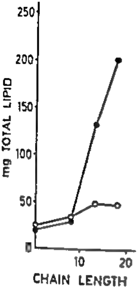
The transport of cholesterol and of total lipid in lipoproteins $S_f<400$ varied independently of each other This is reflected in the percentage lipid composition of the two lipoprotein fractions (Fig 3) Compared to other dietary states MCT as carrier triglyceride produced an increase in the cholesterol part of both lipoprotein fractions while the triglyceride part decreased

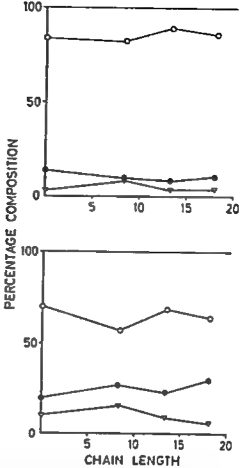
The ratio μmole esterbonds to μmole organic phosphorus was in lipoproteins $S_f>400$ 20—30 while in lipoproteins $S_f<400$ approximately 10

Chromatographic analysis

Fig 4 and 5 show two representative studies with lymph obtained after feeding cholesterol dissolved in either triolein or MCT

In both cases lipoproteins were continuously distributed through the whole elution volume but were concentrated into four classes the K_D values of which are in agreement with previous separations of human plasma lipoproteins (Werner 1966 Hanai *et al* 1968) This is indicated in Table I As the table shows there is disagreement on the exact transition of lipoproteins $S_f>400$ into $S_f<400$ In the present results only the area of transition is indicated because comparison with data obtained from ultracentrifugation gave inconsistent indication on the level of transition The figures show that concentrations of total lipid different lipid classes and of optical density vary separately along the elution volume As in ultracentrifugation the ratio μmole esterbonds/μmole organic phosphorus was about 20—30 in lipoprotein $S_f>400$ and in lipoproteins $400>S_f>20$ about 10





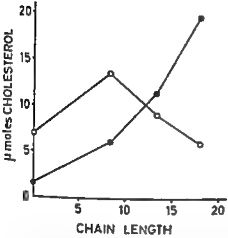


Fig 1 mg total lipid transported in lymph lipoproteins 8 hrs after administration of 50 μmoles radioactive cholesterol This was given dissolved in triglyceride of increasing fatty acid chain length ○——○ indicates lipoproteins $S_f < 400$ and ●——● lipoproteins $S_f > 400$

Fig 2 μmoles cholesterol transported in lymph lipoproteins 8 hrs after administration of 50 μmoles radioactive cholesterol This was given dissolved in triglyceride of increasing fatty acid chain length ○——○ indicates lipoproteins $S_f < 400$ and ●——● lipoproteins $S_f > 400$

Fig 3 Percentage composition of lymph lipoproteins Lymph was sampled during 8 hrs after administration of 50 μmoles radioactive cholesterol dissolved in triglyceride of increasing fatty acid chain length Lipoproteins $S_f > 400$ (Above) and lipoproteins $S_f < 400$ (Below) ○——○ triglyceride (calculated from determinations of ester bonds) ●——● phosphatide and ▽——▽ cholesterol.

We found interestingly in some experiments, that a group of proteins eluted wit' the inclusion volume maintained a ratio of 20—40 The content of organic

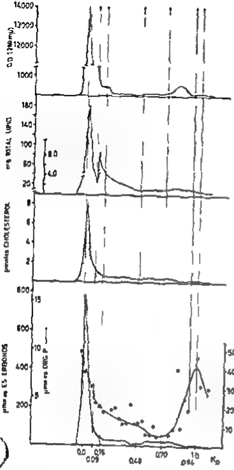


Fig 4

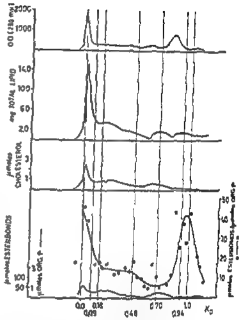


Fig 5

Fig 4 Elution patterns from gel chromatography of lymph sampled during 8 hrs after administration of 50 μmoles radioactive cholesterol dissolved in triolein Top Optical densities at 280 mμ Middle above mg total lipid Middle below ——— μmoles total and μmoles exogenous cholesterol Bottom μmoles esterbonds and ——— μmoles organic phosphorus ●———● ratio μmoles esterbonds to μmoles organic phosphorus

Fig 5 Elution patterns from gel chromatography of lymph sampled 8 hrs after administration of 50 μmoles radioactive cholesterol dissolved in MCT Top Optical densities at 280 mμ Middle above mg total lipid Middle below ——— μmoles total and μmoles exogenous cholesterol Bottom μmoles esterbonds and ——— μmoles organic phosphorus ●———● ratio μmoles ester bonds to μmoles organic phosphorus

phorus in this group was negligible and the content of esterbonds the same as in the latter part of the elution volume Probably the high peak accounts for esters from lipoproteins that have equilibrated with albumin

In agreement with the ultracentrifugal separation, 10 and 40 per cent of total lipid of lipoproteins $S_f > 20$ was transported in lipoproteins $400 > S_f > 20$ when triolein and MCT was used as carrier triglyceride, respectively The low fraction of total lipid transported in this lipoprotein fraction after triolein administration was quantitatively larger than that found when MCT was used (Table II) Total and

TABLE I Lipoprotein classes obtained by agarose gel chromatography as determined by Werner (1966) K_D (1) and by Hanai *et al* (1968), K_D (2) K_D (3) refers to the present experiment

Lipoprotein class	$10^4>S_f>400$	$400>S_f>20$	$20>S_f>0$	Alpha
Molecular weight	3 10^{11}—12 10^6	12 10^6—5 10^6	3 10^6—1 10^6	400 000—150 000
K_D (1)	0 00—0 16	0 16—0 42	0 52—0 63	0 74—0 80
K_D (2)	0 00—0 09	0 09—0 41	0 41—0 75	0 75—0 93
K_D (3)	0 00—0 09 / 0 16	0 09 / 0 16—0 48	0 48—0 70	0 70—0 94

exogenous cholesterol and ester bonds were concentrated in lipoproteins $S_f>400$ with a gradual decrease into lipoproteins $400>S_f>20$ Exogenous cholesterol was found in the whole of this class but not in lipoproteins $S_f<20$

Discussion

Exogenous fat is transported from the intestine partly by chylomicrons *i e* lipoproteins $S_f>400$ and partly by lipoproteins $400>S_f>20$ Particles of the former lipoprotein class have a size of 700 Å—10 000 Å and a molecular weight of 12 10^6—3 10^{11} (Freeman, Lindgren and Nichols 1963) In the body, they are synthesized only by the intestine Particles of the latter class have a size of 300 Å—700 Å and a molecular weight of 5 10^6—12 10^7 (Freeman *et al* 1963) Synthesis takes place in the liver and to a minor extent in the intestine (Havel and Goldfien 1961)

In order to determine whether the particle size distribution of thoracic duct lymph chylomicrons could be influenced by dietary means Zilversmit Sisco and Yokoyama (1966) measured the distribution with density gradient centrifugation after feeding cream or corn oil The mean size was 2500 Å irrespective of the oil fed There was an inverse relationship between size and number of particles However this group pointed out that their method was liable to give inconsistent results for smaller chylomicron particles (Pinter and Zilversmit 1962)

In the present investigation a possible dietary influence on the composition of lipoproteins of the thoracic duct lymph has been examined by agarose gel chroma

TABLE II mg total lipid transported in different liporotein classes Lymph was obtained from rats for 8 hrs after the administration of 50 μ moles radioactive cholesterol with either triolein or MCT as carrier triglyceride The lipoprotein classes were separated by agarose gel chromatography according to K_D values given in Table I

Lipoprotein class	$10^4>S_f>400$	$400>S_f>20$	$20>S_f>0$
Triolein	327 1	27 7	19 0
Triolein	228 8	52 5	38 0
MCT	34 2	24 1	23 4
MCT	58 4	23 2	14 6

tographs This procedure separates the different lipoprotein classes according to their size (Werner 1966 Hanai *et al* 1968) Lipoproteins S_f>400 are mainly found in *the void volume but are continuously distributed along the elution volume* into smaller lipoproteins Thus lipoproteins carrying exogenous lipids are eluted partly with the void volume and partly with the next class of lipoproteins *i e* those of 400>S_f> 20 The chromatographic separations were supplemented by ultracentrifugal separations

The amount of absorbed dietary triglyceride to be transported from the intestine into the lymphatics determines the amount of lipoproteins of both classes formed No increased transport of smaller lipoproteins takes place when small an amount of triglyceride and larger an amount of cholesterol is to be transported from the intestine Instead there is a shift in the lipid composition of lipoproteins transported so that these contain more cholesterol and less triglyceride compared to other dietary states

References

ABELL L L B B LEVY B B BRODIE and F E KENDALL A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity *J biol Chem* 1952 *195* 357—366

ALBRINK M J Lipoprotein pattern as a function of total triglyceride concentrat on of serum *J clin Invest* 1961 *40* 536—544

BIERMANN E L T L HAYES J N HAWKINS A B EWING and F T LINDGREN Particle size distribution of very low density plasma lipoproteins during fat absorption in man *J Lipid Res* 1966 *7* 65—72

...EN ... S JR T Y TORIBARA and ... C WARNER Microdetermination of phosphorus *Anal Chem* 1956 *28* 1756—1758

...ORNWELL D G and F A KRUGER Molecular complexes in the isolation and characterization of plasma lipoproteins *J Lipid Res* 1961 *2* 110—134

...REEMAN N K F T LINDGREN and A V NICHOLS The chemistry of serum lipoproteins *Prog chem Fats Other Lipids* 1963 *6* 216—250

...ANAI T P D S WOOD G D MICHAELS and L W KINSELL Gel chromatography of hyperlipidemic plasma on 2 % agarose *Proc Soc exp Biol (N Y)* 1968 *129* 226—232

...AVEL R J and A GOLDI...
and metabolism of fatty...

HOFMANN A F The beh...
solution *Biochim bioph...*

HOFMANN A F *The function of bile salts in fat absorption* (M D Thesis University of Lund Sweden 1964

PINTER G G and ZILVERSMIT D B A gradient centrifugation method for the determination of particle size distribution of chylomicrons and of fat droplets in artificial emulsions *Biochim biophys Acta* Amst 1962 *59* 116—127

QUARFORDT S H and D W S GOODMAN Heterogeneity in the rate of plasma clearance of chylomicrons of different size *Biochim biophys Acta* (Amst) 1965 *116* 382 385

SCHOTZ M C B ARNESJO and T OLIVECRONA, The role of the liver in the uptake of plasma and chyle triglycerides in the rat *Biochim biophys Acta* (Amst) 1966 *125* 485—495

SNYDER F and N STEPHENS A simplified spectrophotometric determination of ester groups of lipids *Biochim biophys Acta* Amst) 1959 *34* 244 245

SYLVEN, C and B BORGSTROM Intestinal absorption and lymphatic transport of cholesterol in the rat influence of the fatty acid chain length of the carrier triglyceride *J Lipid Res* 1969 *10* 351—355

WERNER M Fractionation of lipoproteins from blood by gel filtration *J Chromat* g 1966 *25* 63—70

ZILVERSMIT D B P H SISCO JR and A YOKOYAMA Size distribution of thoracic duct lymph chylomicrons from rats fed cream or corn oil *Biochim biophys Acta* Amst 1966 *125* 129—135

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Transport of Glucose, Sodium, Chloride and Potassium between the Cerebral Ventricles and Surrounding Tissues in Cats

By

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Abstract

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The ventriculo-aqueductal perfusion technique was used in order to study the mechanism for transport of glucose, sodium, chloride and potassium between the cerebral ventricles and surrounding tissues in cats. The inflow perfusion fluid contained U-^{14}C-D-glucose and unlabelled D-glucose. With D-mannose or D-xylose in the inflow, the unidirectional flux of glucose from the cerebral ventricles was inhibited competitively. Ouabain in the inflow (10^{-7} M or 10^{-6} M) and potassium-free inflow reduced the rate of cerebrospinal fluid formation while the tissues lost potassium and gained sodium and chloride. The presence of ouabain reduced the unidirectional flux of glucose from the cerebral ventricles. The effects increased using higher concentrations of ouabain, up to 5×10^{-5} M. Injections of increasing amounts of ouabain into the internal carotid artery had no effects upon the above mentioned variables. The results support the idea that ouabain sensitive carrier mediated transport of glucose from the cerebral ventricles may be connected with the mechanism for active transport of ions.

Previous investigations on cats have shown that glucose is transported from the cerebral ventricles into surrounding tissues by an ouabain sensitive mechanism, by an ouabain insensitive mechanism and by simple diffusion (Brøndsted 1970). Administration of ouabain intraventricularly resulted in a net loss of potassium from the tissues and a gain in tissue sodium. This suggested a possible linkage in the ouabain sensitive transport of glucose, of sodium and of potassium.

The following investigations were carried out in order to obtain further information about the nature of the transport system. The ventriculo-aqueductal perfusion technique was used. The perfusion fluid contained U-^{14}C-D-glucose to determine the unidirectional fluxes of glucose between the ventricles and surrounding tissues.

In the experiments to be described the following variables were followed during steady state perfusion. The unidirectional fluxes of glucose between the cerebral

ventricles and surrounding tissues, net fluxes of sodium (or chloride) and potassium and the rate of net cerebrospinal fluid (CSF) formation. Concurrently, the concentrations of glucose within the cerebral ventricles and in blood plasma were determined. In one group of experiments the inhibitory effect of mannose or xylose upon glucose transport was studied.

Methods

Animal preparation. Cats of either sex weighing from 3—5 kg were anesthetized with chloralose 70 mg/kg administered i.p. as a 1 % solution. Small doses of a 6 % solution of pentobarbital sodium were given i.p. as necessary. Tracheotomy with intubation was carried out and a polythene catheter was inserted through the right femoral artery into the abdominal aorta. The catheter served for blood sampling and for monitoring of mean arterial blood pressure by means of a mercury manometer. Rectal temperature was maintained at 37.5—38.5° C. In some cats a polythene catheter was implanted central to a ligation of the external carotid artery (Sakata, Hayano and Sloviter 1963) so that substances could be injected directly into the cerebral hemisphere.

The procedures used to establish the perfusion system as well as the analytical techniques have been described in detail elsewhere (Brondsted 1970). In short, they were as follows: fine inflow cannulas (outside diameter 0.35 mm) were inserted into the two lateral cerebral ventricles and an effluent catheter (67 mm in length) was passed under the cerebellum and inserted into the cerebral aqueduct.

Ventriculo-aqueductal perfusion. The basic inflow solution was an artificial CSF (Merlis 1940). If glucose or other sugars were added in different amounts as described in *Results*. Furthermore U-^{14}C-D-glucose (purchased from The Radiochemical Centre, Amersham, England) with a specific activity of 2.7 mC per mmole was added to give an activity of 5 μC per 100 ml solution. The solution was equilibrated with 5 % carbon dioxide to give a final pH between 7.25—7.35.

The rate of inflow was 110 μl/min (S.D. = 0.8, n = 72) and one half of this flow was diverted each of the lateral ventricles.

The outer end of the effluent catheter was in contact with the inside of an almost horizontal placed narrow sampling vessel. The effluent was collected during successive periods of 15 min. Effluent volumes were determined by weighing to the nearest 0.1 mg. Only effluent samples which were clear and free from blood were used for analyses.

The rate of respiration, pulse rate, reflexes etc. were controlled regularly. Materials and instruments were sterilized before coming into contact with tissues or fluids containing glucose.

Analytical techniques. Blood samples were taken every half hour. Two aliquots of a plasma sample and two aliquots of an effluent sample were precipitated with NaOH-$ZnSO_4$ and centrifuged. The supernatants were used for determination of glucose with a glucose oxidase method (Hjelm and de Verdier 1963). Mean recovery of glucose added to effluent samples was 99.2 per cent (S.D. = 1.0, n = 6) and of glucose added to plasma, 100.0 per cent (S.D. = 1.7, n = 7). Twenty determinations of plasma glucose from the same sample gave an average figure of 14.1 mM (S.D. = 0.27). The concentration of glucose in arterial plasma water was found by multiplication of the measured concentration in plasma by a constant (1.08) which was derived from the specific gravity of plasma samples (from six cats) and their content of water. The amount of water was determined by drying at 105° C until constant weight was achieved.

The ^{14}C activity in effluent samples was determined in a Packard Tri-Carb Liquid Scintillator Spectrometer (Model 3003). ^{14}C-carbon dioxide and ^{14}C-lactate which might be formed from ^{14}C-glucose were isolated and determined as previously described. It was found that between 99.5—100.0 per cent of the ^{14}C activity in the effluent could be taken as representative of ^{14}C-glucose and no correction of the measured activity was necessary.

Sodium and potassium in the effluent was determined using an Eppendorf flame photometer. Chloride was determined by electrometric titration (Marius 'Chlor-o-Counter', Bruxelles, Belgium).

The rate of formation of CSF. It has previously been shown (Brondsted 1970) that in the present experimental arrangement the rate of net formation of CSF is equal to the rate of outflow minus the rate of inflow.

Calculations of unidirectional fluxes of glucose. These were carried out as previously described (Brondsted 1970). In short, the model consists of two compartments (the cerebral ventricles

and the plasma/brain compartment) separated by a homogenous membrane Interactions between solute flows as well as exchanges across the blood brain barrier were ignored It is assumed that ^{14}C-glucose moves from the ventricles into plasma and brain only During steady state the amount of ^{14}C-glucose in the ventricles remains constant and the rate of inflow of tracer will therefore be equal to the rate of loss from the system plus the rate of collection of tracer In other words the unidirectional flux of ^{14}C glucose ($J^{*}_{v\ pb}$ cpm/min) will be equal to the amount of tracer entering the inflow cannulas per min minus the amount leaving the effluent catheter per min

In addition to the already mentioned symbol ($J^{*}_{v\ pb}$) the following symbols are used $J_{v\ pb}$ is the unidirectional flux of glucose from the ventricles (µmoles/min), $\overline{C}_v$ (mM) is the mean ventricular concentration of glucose (the arithmetical mean of the concentration in the inflow and in the outflow was used), $\overline{C}^{*}_v$ (cpm/ml) is the mean ventricular concentration of ^{14}C-glucose (assuming an exponential decrease of concentration within the system $\overline{C}^{*}_v = 0\ 37 \times$ inflow concentration $+ 0\ 63 \times$ outflow concentration) If it is assumed that

$$J_{v\ pb}/\overline{C}_v = J^{*}_{v\ pb}/\overline{C}^{*}_v$$

then the unidirectional flux of glucose from the ventricles can be derived from this equation

The net flux of glucose between the two compartments was determined as the amount of glucose leaving the effluent catheter per min minus the amount entering the inflow cannulas per min (N µmoles/min) The net fluxes of potassium sodium and chloride were determined in the same way The net fluxes were called positive if they were directed from the tissues into the ventricles The unidirectional flux of glucose from plasma and brain ($J_{pb\ v}$ µmoles/min) is the sum of N and $J_{v\ pb}$

Results

Steady state After 45—60 min of perfusion the ^{14}C D glucose in the effluent remained constant within 1 9 per cent When the inflow solution was altered in various ways during steady state it usually lasted 15 min or less before a new steady state was approached

Competition between the related sugars D glucose D mannose and D xylose The outflow concentrations of D glucose always increased when D mannose or D xylose were added to the perfusate and the unidirectional flux of D glucose from the cerebral ventricles into plasma and brain was significantly reduced (Table I) Transport by simple diffusion (around 50 per cent of total unidirectional flux Brondsted 1970) was subtracted before the fluxes were compared The effects were reversible

The unidirectional transport of glucose in the opposite direction *i e* from plasma and brain into the cerebral ventricles (not shown) was slightly increased but this was probably due to small increases in blood glucose concentrations which were observed simultaneously

Increasing concentrations of ouabain intraventricularly Ouabain in a concentration of 5×10^{-5} M in the inflow solution reduces the unidirectional flux of glucose between the cerebral ventricles and surrounding tissues (Brondsted 1970) Furthermore the rate of formation of CSF decreases and the amount of effluent potassium sampled per min increases while that of sodium decreases *i e* the tissues lose potassium and gain sodium It was therefore of interest to see if these effects would be augmented when ouabain was administered intraventricularly in increasing concentrations and to see if chloride movement could be altered in a manner similar to that of sodium

The experiment depicted in Fig 1 shows the effects of increasing concentrations of ouabain in the inflow upon some of these variables The first steady state period

TABLE I Carrier mediated unidirectional flux of D glucose from the cerebral ventricles (control periods) and inhibition of the flux upon addition of D mannose or D xylose to the inflow (11 mM) Results are mean values from 2—4 15 min sampling periods during steady state (μmoles/min) t test p<0 005 for all experimental periods as compared with their control periods Mean ventricular concentrations of glucose 4—7 mM with the lowest values during control periods

Experiment no	1	2	3
Control period	0 06		0 08
D mannose	0 03		0 06
Control period		0 04	0 08
D xylose		0 03	0 06
Control period		0 04	0 08

lasted for half an hour (bottom scale) There was no ouabain in the inflow (top scale) during this period which served as a control During the following half hour intervals the inflow contained ouabain in increasing concentrations When ouabain in a new concentration had been administered 15 min were allowed for a new steady state to be approached and so forth The inflow solution contained no unlabelled glucose

Small effects of ouabain appeared at 10 ⁷ M—10 ⁶ M They became clearly visible at 10 M and were even more pronounced at 5×10 ⁶ M The rate of net CSF formation, the unidirectional flux of glucose from the cerebral ventricles and the net flux

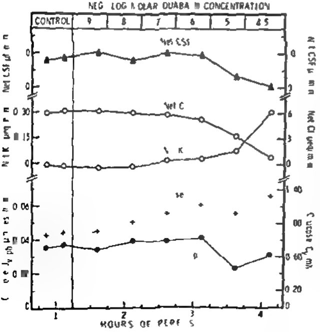

Fig 1 Effects of perfusing the first three cerebral ventricles with increasing amounts of ouabain in the inflow (top scale) The concentration was changed every half hour (bottom scale) Five parameters were followed (broken ordinates) the rate of net CSF formation (▲—▲) net fluxes of chloride (○—○ right ordinate) and potassium (○—○ left) and the unidirectional flux of glucose for the ventricles J_{gl} (●—● left) together with the mean ventricular concentration of glucose $\bar{C}_v$ (+ + right) Net fluxes are positive from plasma and brain into the ventricles

TABLE II Ouabain-sensitive net fluxes of glucose, sodium and potassium (μeq/min) in a series of experiments which have been arranged in an increasing order of magnitude with respect to the numerical values of the ouabain sensitive net fluxes of glucose (first column). Net fluxes from the tissues into the cerebral ventricles are positive. Results are mean values from 3—12 15-min sampling periods during steady state. The last column gives the ratio of ouabain sensitive net fluxes of sodium to those of potassium

Net glucose	Net Na	Net K	Ratio net (Na)/K
0.01	1.25	−0.13	10:1
0.01	0.66	−0.14	5:1
0.01	0.67	−0.16	4:1
0.02	0.70	−0.21	3:1
0.03	1.25	−0.17	7:1
0.03	1.07	−0.11	10:1
0.04	1.07	−0.15	7:1
−0.06	1.05	−0.16	[illegible]:1
−0.06	1.07	−0.17	6:1
0.06	1.44	−0.15	10:1
0.06	1.11	−0.13	9:1

of chloride from the tissues decreased while that of potassium increased. There was a small deficit in chloride in the inflow causing a loss of chloride from the tissues. This loss was diminished during ouabain administration. Thus, the tissues lost potassium and gained chloride.

A decrease of the unidirectional flux of glucose from the tissues into the ventricles was also seen at 10^{-5} M (not shown) but this change of flux was of minor interest because of its association with fluctuating blood sugar values.

Ouabain sensitive net fluxes of glucose, sodium and potassium. The ouabain sensitive net fluxes were computed by subtraction of the net flux during ouabain administration (10^{-4} M in the inflow) from the net flux before ouabain administration in the same experiment. A comparison of these fluxes are seen in Table II. The direction of the ouabain sensitive net fluxes of glucose was from the tissues into the cerebral ventricles (positive values) except in two cases where the mean ventricular concentration of glucose was higher than the concentration in blood plasma, whereby the fluxes became negative (first column of Table II). The direction of the ouabain sensitive net fluxes of sodium was from the tissues into the cerebral ventricles while ouabain sensitive net fluxes of potassium had the opposite direction. The magnitude of these fluxes were essentially the same for each of the cations and they did not seem to be influenced by the magnitude of, or the direction of, the ouabain sensitive net fluxes of glucose. The last column of Table II shows that if one molecule of potassium were absorbed from the perfusate by an ouabain sensitive mechanism, 3—10 molecules of sodium would pass in the opposite direction.

TABLE III Reversible effects of potassium free inflow upon the rate of net CSF formation and upon net fluxes of potassium and sodium Net fluxes from plasma and brain into the perfusate are positive Unidirectional fluxes of glucose from the cerebral ventricles ($J_{v\,pb}$) and from plasma and brain ($J_{pb\,v}$) are shown together with the concentrations of glucose within the cerebral ventricles ($\overline{C_v}$) and in plasma water (C_{pw}), respectively 1—2 15 min sampling periods during steady state were used

	Net CSF (µl/min)	Net K⁺ (µeq/min)	Net Na (µeq/min)	$\overline{C_v}$ (mM)	$J_{v\,pb}$ (µmoles/min)	C_{pw} (mM)	$J_{pb\,v}$ (µmoles min)
Control	6 5	0 02	0 98	2 7	0 10	18 7	0 49
K⁺ free	3 8	0 16	0 20	2 9	0 11	17 2	0 51
Control	5 8	—0 02	0 70	2 6	0 10	15 3	0 44
K⁺ free	2 7	0 17	—0 14	2 6	0 10	15 0	0 41
Control	5 5	—0 03	0 55	2 5	0 10	15 3	0 42

Potassium free inflow perfusion fluid The effects of perfusing the cerebral ventricles with this fluid, to which sodium was added in amounts equivalent to the lack of potassium are seen in Table III In this experiment there were two experimental periods with potassium free inflow and three control periods with normal perfusion fluid The rate of net CSF formation was approximately halved during the experimental periods and it returned promptly to normal values during control periods ilarly, the net fluxes of sodium from plasma and brain were reduced while the fluxes of potassium were increased Both of these effects were easily reversed during control periods and all the effects were seen during the first 15 min sampling riod after the new perfusion fluid had been introduced into the cerebral ventricles The outflow concentrations of sodium were 134—136 µeq/ml during all periods The outflow concentrations of potassium were 2 70—2 93 µeq/ml during control periods and 1 37—1 44 µeq/ml during experimental periods i e the perfusate was far from being potassium free after its passage through the cerebral ventricles

As the magnitude of the transport of glucose by simple diffusion from blood to CSF is not known the values in Table III for unidirectional fluxes of glucose from the perfusate to surrounding tissues and the unidirectional fluxes in the opposite direction both include transport by simple diffusion and carrier mediated transport None of the fluxes were altered during periods with potassium free inflow (Around 35 per cent of the unidirectional fluxes from the ventricles (Table III) is transport by simple diffusion (Brondsted 1970

Ouabain injections into the left internal carotid artery By implantation of a polythene catheter central to a ligation of the left external carotid artery it was possible to inject ouabain into the internal carotid artery While the cerebral ventricles were perfused in the usual way two ml of an isotonic solution of sodium chloride was slowly injected into the artery This served as the control period During the following half hour intervals two ml of this solution containing increasing con

TABLE IV Injections of ouabain into the left internal carotid artery while the cerebral ventricles were perfused with normal perfusion fluid containing glucose and ^{14}C-glucose. Two ml of an isotonic solution of sodium chloride was injected (control period one half hour). Subsequently, injections every half hour of this solution containing ouabain 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M (ouabain period). Mean values from two 15 min sampling periods during steady state from each injection period were used. Symbols are the same as in Table III.

	Net CSF (µl/min)	Net K⁺ (µeq/min)	Net Na (µeq/min)	$\overline{C_v}$ (mM)	$J_{v\,pb}$ (µmoles/min)	C_{pw} (mM)	$J_{pb\,v}$ (µmoles/min)
Control	7.3	0	0.76	6.7	0.18	14.3	0.23
Ouabain	6.0	0	0.76	6.8	0.19	15.3	0.25

centrations of ouabain were injected. These periods should be long enough to see an effect of ouabain upon glucose fluxes in the cerebral ventricles. After two hours of ouabain injections the animal had received 0.16 mg of ouabain (LD_{50} for cats = 0.10 mg/kg). During the following period with ouabain injection (10^{-3} M), the respiration stopped while the heart was still beating for some time.

The results from one experiment which is representative for three experiments are seen in Table IV, where the control period is compared to the total ouabain period of two hours. At no time during the injections were there any effects of ouabain upon the rate of CSF formation, or upon net fluxes of sodium or potassium between the cerebral ventricles and plasma/brain. No effects, either, were seen upon the unidirectional fluxes of glucose which followed the concentrations of glucose within the cerebral ventricles and in plasma water. The small reduction in the rate of CSF formation was not seen in the other experiments.

Discussion

Competition phenomena. Table I in the previous section clearly shows a reduced transport of D glucose from the first three cerebral ventricles into surrounding tissues when either D mannose or D xylose was added to the perfusate. The three sugars have similar structures and approximately the same molecular weights. D mannose, like D glucose, is rapidly metabolized by brain *in vivo* (Maddock, Hawkins and Holmes 1939) and *in vitro* while xylose is not metabolized *in vitro* (Page 1937). Consequently, xylose must affect a transport system which is probably common to the three sugars. The observed competition between glucose and the two other sugars thus offers a further indication of a mobile carrier operating in the system (Stein 1967) in addition to the saturation phenomenon described in an earlier report (Brondsted 1970).

These phenomena are similar to those reported for sugar transport in 1) the perfused cerebral ventricular system in the rabbit (Bradbury and Davson 1964), 2) th subarachnoid space in dogs (Fishman 1964, Atkinson and Weiss 1969), 3) the bl

brain barrier in dogs (Crone 1965), cats (Eidelberg Fishman and Hams 1967), mice and rats (LeFevre and Peters 1966, Bidder 1968), and in rabbits (Agnew and Crone 1967) Furthermore, *in vitro* experiments have shown saturation and competition phenomena between sugars in brain tissue (Gilbert 1965) and in the choroid plexus (Csaky and Rigor 1964)

There are three main sites to which glucose in the perfusate can go cerebral capillary blood brain parenchyma (neurones and glia cells) and the epithelium of the choroid plexus It is not possible to state which of these places are the most important in exhibiting the described phenomena

Increasing concentrations of ouabain intraventricularly Ouabain 10^{-5} M in the inflow clearly reduced the unidirectional flux of glucose from the cerebral ventricles (Fig 1) and a small effect was seen at 10^{-7} M—10^{-6} M Similar concentrations of ouabain altered the other variables (net CSF formation and net fluxes of chloride and potassium) and the effects increased similarly with increasing concentrations of ouabain As ouabain inhibits cation activated 'transport ATPase' the findings support the idea that there may be some connection between the transport of glucose and of potassium and/or sodium

Chloride was affected in a way similar to that of sodium, *i e* there was a relative gain of chloride in the tissues during ouabain perfusion Ames Higashi and Nesbett (1965) found an increase in potassium concentration in newly formed choroid plexus fluid when ouabain (10^{-4} M) was applied topically but there was no effect upon sodium or chloride It is possible that the effects of ouabain upon sodium and chloride in the perfusion system is confined to the brain parenchyma

The effect of ouabain intraventricularly upon the rate of CSF formation was concentration dependent within the range of 10^{-7} M—5×10^{-5} M This confirms the observations by Vates Bonting and Oppelt (1964) The net transport of chloride and of potassium and the unidirectional flux of glucose were not altered by the low concentrations of ouabain (10^{-8} M—10^{-6} M)

Ouabain sensitive net fluxes of glucose sodium and potassium A comparison of these fluxes (Table II showed no relationship between ouabain sensitive net fluxes of glucose and those of the cations the latter fluxes being largely the same whether the glucose fluxes were about 0 01 μmole/min or about 0 06 μmole/min or whether their direction was from the tissues into the perfusate or the opposite This observation does not exclude a coupling between ouabain sensitive unidirectional fluxes of glucose sodium and/or potassium The perfusion system is very complex and ouabain sensitive transport at different sites may not be equally important for each of the three molecules Information about this point is not available

The ratio of ouabain sensitive net fluxes of sodium to those of potassium showed that if one molecule of potassium passed from the perfusate into surrounding tissues 3—10 molecules of sodium would pass in the opposite direction This finding may be indicative of some degree of coupling between these ions when transported by an ouabain sensitive mechanism A similar coupling has been proposed by Bradbury and Stulcova (1969) In addition to other tissues it may also exist in human erythrocytes

(Garrahan and Glynn 1967) But again the present system is too complex to state the exact ratio, if any such coupling exists at all

Potassium free inflow perfusion fluid The effects of this fluid upon net CSF formation and upon net fluxes of sodium and potassium were similar to the effects obtained with ouabain (Table III and Fig 1) The inhibition of CSF formation is in concordance with the *in vitro* observation that potassium free medium inhibits the sodium potassium activated ATPase activity in excised cat choroid plexus (Vates *et al* 1964) and with the close relationship found between inhibition of that activity *in vitro* and inhibition of CSF formation *in vivo* when ouabain is administered to the incubation medium and intraventricularly, respectively (Vates *et al* 1964)

There were no effects upon the unidirectional fluxes of glucose as could have been expected if they were coupled to the function of the 'transport ATPase' This might be due to the difficulty in obtaining a sufficiently low concentration of potassium in the extracellular fluid of the brain (Davson 1967) The fact that potassium free perfusion fluid inhibits CSF formation only, while ouabain inhibits CSF formation and the transport of glucose as well points to brain tissue being the important site for ouabain sensitive transport of glucose

Ouabain injections into the left internal carotid artery It was hoped that this method of administration of ouabain would affect some of the variables studied during ventriculo-aqueductal perfusion before serious poisoning of the animal occurred Ouabain seems to pass the blood brain barrier fairly rapidly at least at the site of the respiration center Within two min after injection of 10^{-3} M ouabain (total 1 6 mg) the respiration stopped while the heart continued its beating regularly and at a normal rate for some minutes afterwards Similar symptoms were described by Vates *et al* (1964) who used high concentrations of ouabain intraventricularly in cats By intravenous administration of other cardiac glycosides they obtained a slight inhibition of CSF formation

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References

AGNEW W F and C CROSE Permeability of brain capillaries to hexoses and pentoses in the rabbit *Acta physiol scand* 1967 *70* 168—175

AMES A III K HIGASHI and F B NESBETT Relation of potassium concentration in choroid ... (Lond) 1965 *181* 506—515

... fluid glucose transfer in the

... Configurational aspects *J*

... eatinine and certain monosaccharides between blood and fluid perfusing the cerebral ventricular system of rabbits *J Physiol* (Lond) 1964 *170* 195—211

BR ... responsible for the stability of the con... ysiol (Lond) 1969 *204* 138P—1

BR ... transport of glucose from the

CRONE, C, Facilitated transfer of glucose from blood into brain tissue *J Physiol* (Lond) 1965 *181* 103—113
CSAKY, T Z and B M RIGOR, A concentrative mechanism for sugars in the choroid plexus *Life Sci* 1964 *3* 931—936
DAVSON, H, *Physiology of the cerebrospinal fluid* London, Churchill 1967 pp 212—213
EIDELBERG, E, J FISHMAN and M L HAMS, Penetration of sugars across the blood brain barrier *J Physiol* (Lond) 1967 *191* 45—57
FISHMAN, R A, Carrier transport of glucose between blood and cerebrospinal fluid *Amer J Physiol* 1964 *206* 836—844
GARRAHAN, P J and I M GLYNN, The stoicheiometry of the sodium pump *J Physiol* (Lond) 1967 *192* 217—235
GILBERT, J C, Mechanism of sugar transport in brain slices *Nature* (Lond) 1965 *205* 87—88
HJELM, M and C-H DE VERDIER, A methodological study of the enzymatic determination of glucose in blood. *Scand J clin Lab Invest* 1963 *15* 415—428
LEFEVRE P G and A A PETERS Evidence of mediated transfer of monosaccharides from blood to brain in rodents *J Neurochem* 1966 *13* 35—46
MADDOCK, S, J E HAWKINS and E HOLMES, The inadequacy of substances of the 'glucose cycle' for maintenance of normal cortical potentials during hypoglycemia produced by hepatectomy with abdominal evisceration *Amer J Physiol* 1939 *125* 551—565
MERLIS, J K, The effect of changes in the calcium content of the cerebrospinal fluid on spinal reflex activity in the dog *Amer J Physiol* 1940 *131* 67—72
PAGE, I H, *Chemistry of the Brain* London, Baillière, Tindall & Cox 1937 p 402
SAKATA, K, S HAYANO and H A SLOVITER, Effect on blood glucose concentration of changes in availability of glucose to the brain *Amer J Physiol* 1963 *204* 1127—1132
STEIN, W D, *The movement of molecules across cell membranes* New York, Academic Press. 1967 pp 127—128
VATES T S, S L BONTING and W W OPPELT, Na K activated adenosine triphosphatase formation of cerebrospinal fluid in the cat *Amer J Physiol* 1964 *206* 1165—1172

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Release and Uptake of Dopamine in Isolated Granules from a Human Carotid Body Tumour

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Abstract

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Isolated granules from a human carotid body tumour incubated in 130 mM K phosphate pH 7 5 release dopamine (DA) with a half time of 23 min at 37° C Addition of DA $2 \times 10^{-?}$ M to [illegible] urther [illegible] effect is [illegible] (NA) [illegible] a concentration of 10 [illegible] M

The chromaffin reaction in the glomus cells of the carotid body was first shown by Smith (1924) De Kock (1954) later observed two different types of chromaffin cells in the cells of the carotid body Granules similar to those in the adrenal medulla and presumably containing catecholamines (CA) were shown by Lever and Boyd (1957) and Ros (1957) The presence of CA in the carotid body of calf and pig has been reported by Muscholl *et al* (1960) and Rahn (1961) and in man by Pryse Davies *et al* (1964) and Niemi and Ojala (1964) Different types of glomus cells containing catecholamines including DA were found in the carotid body of the cat and rabbit by Chiocchio *et al* (1966) Dearnaley *et al* (1968), Helpap and Hempel (1968) and Morita *et al* (1969)

In the present communication it is shown that isolated granules from a human carotid gland tumour release and take up DA in the same way as DA granules in the adrenal medulla (Lishajko 1969)

Material and Methods

[illegible]

fuged at 50 000 × g for 30 min The tube was washed with phosphate buffer and the high speed sediment resuspended in 100 ml 130 mM K phosphate Tubes containing [illegible] ml each of this sus

column for all three amines is about 70 per cent

Radioactivity was measured in a Packard Tri carb liquid scintillation counter C^{14} labelled DA specific activity 7 07 mC/m mole was obtained from New England Nuclear Corporation ATP was obtained from Sigma Chemicals

Results

Amine analysis in whole tissue

Analysis of the carotid body tumour tissue yielded 8 2 μg DA, 3 2 μg A and 2 0 μg NA, calculated per g tissue, or 61, 24 and 15 per cent respectively of total amines (corrected for loss during adsorbtion on alumina) The results are in a fairly good agreement with the findings of Chiocchio *et al* (1966), for the cat carotid body The average proportions found in their specimens were 56 % DA, 7 8 % A and 36 % NA

Release and reuptake of DA in isolated granules

The high speed sediment after homogenization of the tissue suspended in 130 mM K phosphate buffer pH 7 5 released 50 per cent of the DA content during incubation for 23 min at 37° C, giving a concentration of free DA in the supernatant of $1\ 4 \times 10^{-6}$ M Incorporation of labelled ^{14}C-DA during 10 and 30 min incubation at 37° C is seen in Fig 1 On addition of DA 2×10^{-5} M to the suspension together with ^{14}C-DA net uptake of DA occurs Addition of ATP-Mg^{2+} 2 mM in the presence of about $1\ 4 \times 10^{-6}$ M DA in the supernatant also causes net uptake of DA but reduces the proportion of incorporated ^{14}C DA A higher concentration of DA, 2×10^{-5} M added together with ATP-Mg 2 mM to the incubation medium, causes an accumulation of DA of about [illegible] times in the granules (Fig 1) Furthermore almost all DA found in sediment was equilibrated with ^{14}C DA and nonlabelled external DA after 30 min as evidenced by the specific activity ratio in sediment and supernatant (^{14}SED/^{14}SU) which approached the value 1 The uptake of DA in the sediment after addition of ATP alone or together with DA 2×10^{-5} M suggests that granules were initially partially depleted Reserpine 10 [illegible] M strongly decreases the incorporation of DA in granules, suggesting that this is of specific character

NA and A each at a concentration of 10^{-5} M reduce incorporation of labelled and unlabelled DA by about 40 per cent suggesting that these amines compete with DA uptake

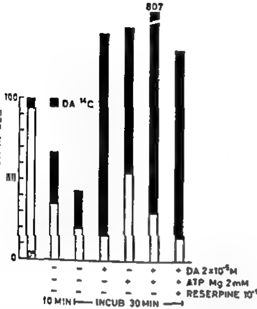

Fig 1

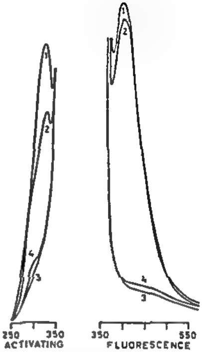

Fig 2

Fig 1 Human carotid body tumour granules incubated in 130 mM K phosphate buffer pH 7 5 at 37° C Additions as indicated Free DA in medium about $1\ 5 \times 10^{-6}$ M Ordinate Remaining and accumulated DA after incubation in per cent of original amount

Fig 2 Activating DA spectra in glomus cell granules corresponding to 0 06 μg (curve 2 left) and fluorescence DA spectra in whole human carotid body tumour tissue aliquot of eluate after Al_2O_3 column) corresponding to 0 4 μg DA (curve 2 right) and standard DA (curve 1, left and right) after oxidation and transformation to the fluorophores 1 authentic DA 2 sediment or tissue 3 DA blank 4 sediment or tissue blank. Activating 340 mμ and fluorescence 395 mμ (non-corrected) wavelength Aminco-Bowman instrument

Identification of DA in sediment and in total tissue

Aliquots of granule bound DA as well as in the eluate from total carotid body tissue extract (after oxidation and transformation to a fluorophores) was used for identification of DA with authentic DA as a reference (Fig 2)

Discussion

The present experiments have demonstrated that release and uptake of DA in isolated granules from human carotid tumour tissue follow the same general pattern as in adrenal medullary granules from various species Thus the half time for rele[illegible] DA from sheep medullary granules at 37° C was about 20 min (Lishajko [illegible] compared with 23 min for the granules studied here The ATP Mg [illegible] uptake exceeded any values previously observed possibly indicatin[illegible]

cells were largely depleted at the beginning of the experiments In this respect the DA granules differ from the NA or A granules in chromaffin cells which lack the ability to take up amines above their initial content The existence of a high proportion of specific DA cells in the carotid body suggests a physiological role of DA in this organ possibly connected with the function of the chemoreceptors, acting on respiration and circulation (Jacobs and Comroe 1968) The remarkable sensitivity of this reflexogenic zone to nicotine (Heymans *et al* 1930) or acetylcholine (Euler 1938) might be connected with a release of amines from the catecholamine cells in the organ An amine releasing action of this kind has recently been demonstrated for DA in the adrenal medulla (Lishajko 1970)

I am indebted to professor C A Hamberger Karolinska Hospital, Stockholm, for placing the tumour at my disposition The research reported in this document has been sponsored in part by the Swedish Medical Research Council project no B70 14X 97 06B and Knut and Alice Wallenberg Foundation

References

CARLSSON A and B WALDECK A fluorimetric method for the determination of dopamine (3 hydroxytyramine) *Acta physiol scand* 1958 *44* 293—298

CHIOCCHIO S R A M BISCARDI and J H TRAMEZZANI Catecholamines in the carotid body of the cat *Nature* (Lond) 1966 *212* 834—835

DEARNALEY D P M FILLENZ and R I WOODS The identification of dopamine in the rabbit s carotid body *Proc roy Soc B* 1968 *170* 195—203

EULER U S v Reflektorische und zentrale Wirkung von Kaliumionen auf Blutdruck und Atmung *Skand Arch Physiol* 1938 *80* 94—123

EULER U S v and F LISHAJKO Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—355

HELPAP B and K HEMPEL Autoradiographische Untersuchungen mit ^{3}H DOPA zum Catecholamin Stoffwechsel des Karotiskorperchens *Verh dtsch path Ges* 1968 *52* 464—469

HEYMANS C J J BOUCKAERT and L DAUTREBANDE Sinus carotidien et réflexes respiratoires II Influences respiratoires reflexes de l acidose de l alcalose de l anhydride carbonique de l ion hydrogène et de l anoxemie Sinus carotidiens et echanges respiratoires dans les poumons et au dela des poumons *Arch int Pharmacodyn* 1930 *39* 400—448

JACOBS L and J H COMROE JR Stimulation of the carotid chemoreceptors of the dog by dopamine *Proc nat Acad Sci* (Wash) 1968 *59* 1187—1193

KOCK L L DE Histology of the carotid body *Nature* (Lond) 1954 *167* 611—612

LEVER J D and J D BOYD Osmiophile granules in the glomus cells of the rabbit carotid body *Nature* (Lond) 1957 *179* 1082—1083

LISHAJKO F Release reuptake and net uptake of dopamine noradrenaline and adrenaline in isolated sheep adrenal medullary granules *Acta physiol scand* 1969 *76* 159—171

LISHAJKO F Dopamine secretion from the isolated perfused sheep adrenal *Acta physiol scand* 1970 *79* 405—410

MORITA E S R CHIOCCHIO and J H TRAMEZZANI Four types of main cells in the carotid body of the cat *J Ultrastruct Res* 1969 *28* 399—410

MUSCHOLL E RAHN K H and M WATZKA Nachweis von Noradrenalin im Glomus caroticum *Naturwissenschaften* 1960 *14* 325

NIEMI M and K OJALA Cytochemical demonstration of catecholamines in the human carotid body *Nature* (Lond) 1964 *203* 539—540

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From the Department of Physiology, University of Turku Finland

Testosterone Metabolism by Gastrointestinal Microsomes

By

M.-P. Harri, K. Hartiala, W. Nienstedt and H. A. Salmi

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Abstract

Harri, M.-P., K. Hartiala, W. Nienstedt and H. A. Salmi. *Testosterone metabolism by gastrointestinal microsomes.* Acta physiol. scand. 1970. *79.* 537—540.

In incubation studies with the microsomal fraction of various tissues of the rat the ability to metabolize testosterone, determined as the decrease in $NADPH_2$ absorbance, was found to be rather high in various gastrointestinal tissues but lower in several non-gastrointestinal tissues. In one case some of the numerous metabolites were identified with hepatic, ileal mucosal and heart tissues. All of them apparently produce at least 4-androstenedione, 5α-androstanedione, and 17β-hydroxy-5α-androstan-3-one.

It has been known for many years that in addition to the liver the intestinal wall also plays an active role in the metabolism and deactivation of different steroids (Lehtinen, Nurmikko and Hartiala 1958, Kreek *et al.* 1963). Similar activity has been demonstrated in many other organs, especially in the liver (Mosebach *et al.* 1966, Schriefers, Cremer and Otto 1967), but the relative ability of different tissues to metabolize the steroids has been studied only on a minor scale (Mosebach *et al.* 1966, El Attar and Dirscherl 1965, Sholiton, Marnell and Werk 1966).

It has been shown in several studies that the metabolism of steroids in the liver occurs in the microsomes and that the microsomal enzymes require $NADPH_2$ as cofactor. The hydroxylating enzymes require, in addition, the presence of molecular oxygen, as has been shown with C_{21} steroids by Omura and Sato (1964).

In this investigation the testosterone-metabolizing activity of the microsomal fraction of various gastrointestinal tissues of the rat has been measured by determining the consumption of NADPH. For comparison some other tissues have also been tested.

Material and methods

Wistar rats of both sexes were used in the study. The activity of the microsomal oxidating $NADPH_2$ in the presence of testosterone was studied using the following tissu gastric, duodenal and ileal mucosa, kidney, cardiac muscle, skeletal muscle, spleen

Isolation of the microsome fraction

The method described by Leybold and Staudinger (1959) was employed

The rats were killed by a blow on the neck The tissues were removed rapidly and transferred into an ice-cold SVT mixture containing sucrose (8 59 g), EDTA (0 200 g) and TRIS (0 242 g) in distilled water (100 ml) The stomach and the intestines were opened with scissors and rinsed with cold saline The mucosa was immediately stripped off while keeping the samples on glass sheets placed on ice All tissue specimens were then homogenized into the SVT … an ice water mixture

For … ntrifuge was employed The homo… 000 × g The supernatant was decan… min at 112 000 × g A homogenous brown pink glacial precipitate the microsome fraction was sedimentated It was rehomogenized into an ice cold 0 066 M phosphate buffer (pH 7 0) If storage was necessary the sedimentated microsomal fraction was kept overnight at −15° C before homogenization According to Leybold and Staudinger (1959) freezing has no effect on the activity

Determination of the enzyme activity

The method of Leybold and Staudinger (1959) was applied in a slightly modified form The enzymatic activity of the microsomes was determined optically as the decrease in the absorbance of $NADPH_2$ acting as a hydrogen donor in the testosterone metabolism A Beckman DU spectrophotometer was used The temperature was maintained constant at 37° C (± 0 05° C) with circulating water The reactions were carried out in a 0 066 M phosphate buffer at pH 7 0 Testosterone (Fluka), $NADPH_2$ and microsomes were transferred into the cuvettes at the beginning of the reaction In order to improve the solubility of testosterone propyleneglycol was used Its final concentration was 2 3 %, an amount which cannot inhibit the enzymatic reactions The final volume in the cuvette was 3 ml

Testosterone was added in 96 % ethanol and its final concentration was 0 8 % The amount of testosterone was 8×10^{-5} M, which causes opalescence Due to the large surface of the undissolved testosterone the solution apparently remains saturated even when testosterone is metabolized The absorbance of testosterone was studied as a function of time and the slight increase in the extinction was corrected in the final results

$NADPH_2$ (Sigma Co) was used in a concentration of 10^{-4} M It was dissolved in a phosphate buffer at pH 7 0 immediately before the determination of the activity

The nitrogen determinations were made according to the usual micro Kjeldahl procedure

The absorbance was measured at the beginning and after 6 min at 366 nm The extinction caused by the spontaneous oxidation of $NADPH_2$ (determined without added testosterone) was subtracted from the decrease in the absorbance The spontaneous oxidation of $NADPH_2$ was determined separately for each sample The steroids do not affect the dispersion of the microsomes or the absorbance caused by them (Leybold and Staudinger 1959)

Partial characterization of the metabolites

In one case the microsomes from the liver ileal mucosa and heart of a male rat were further studied in order to identify some of the main metabolites The method of fractionating the sample into lipid free steroid steroid sulphate and steroid glucuronide fractions and … of further characterizing … of years (Nienstedt and H… radioactive testosterone … fractions was counted … steroid sample was … silica gel chromatograph… and an exposition time of about 2 weeks Authentic reference steroids (20—30 µg) were … ed to the samples prior to the bidimensional run After radioautography the plates were stained with a modified Liebermann Burchard reagent and the coloured spots matched with the radioautographic spots on the film

Results

The results of the determinations of the decrease in absorbance are given in Table I

In the experiment in which the metabolites were studied further over 99 per cent of the total radioactivity was found in the free steroid fraction with the single

TABLE I The decrease in the absorbance of NADPH$_2$ (Δ F) at 366 nm 6 min after adding testosterone to rat microsomal incubations calculated to 1 mg of microsomal nitrogen

Tissue	Number of animals	Δ E $\times 10^{3}$	
		Mean	Range
Liver	8	60	(52—74)
Ileal mucosa	8	58	(49—76)
Duodenal mucosa	12	54	(49—70)
Gastric mucosa	8	46	(41—52)
Kidney	8	37	(32 40)
Skeletal muscle	4	22	20—26)
Spleen	4	18	(15 20)
Brain	4	13	10 14)
Heart muscle	4	8	(6 9

exception of the liver, which had 96 8 of the radioactivity in the free steroid fraction and 2 3 per cent in the 'steroid glucuronide fraction

The radioautograms of the microsomal fraction of duodenal mucosa and liver are shown in Fig 1 Quantitative differences are apparent Qualitatively however, the metabolites that could be identified were the same in all three tissues

Unchanged testosterone (A) which forms the largest spot on the ileal mucosal plate had almost disappeared from the liver plate The largest group on the liver plate (H) was that of polyfunctional steroids (containing three or more free functional groups) On the ileal mucosal plates only a trace of the radioactivity was found in this area 4 Androstenedione (B) 5α androstanedione (C) and 17β hydroxy 5α-androstan 3 one (D) were also found The spot marked E is probably a saturated diol of the 5α series

Discussion

Rather strong testosterone metabolizing activity by all the gastrointestinal tissues studied is suggested by the results shown in Table I The activity seems to be somewhat lower in non gastrointestinal tissues

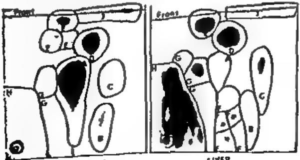

Fig 1 Unconjugated metabolites of testosterone formed by duodenal mucosal and l somes Radioautograms after b dimensional t

In Fig 1 the aim was not to give quantitative results, but only to suggest possible metabolic routes of testosterone metabolism Quantitatively the results in Table I and Fig 1 seem to differ greatly However, as is suggested by Fig 1, the decrease in NADPH adsorbance is probably not affected by testosterone alone, but also by its metabolites Also, the incubation for Fig 1 was made on the basis of the tissue wet weight, with the result that the liver tissue had, in the incubation, about ten times as much microsome nitrogen as the ileal mucosal tissue

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References

EL ATTAR T and W DIRSCHERL Der initiale Stoffwechsel von (4 ^{14}C) Testosteron in der Ratte IV In Niere Hoden und Muskulatur *Hoppe Seylers Z physiol Chem* 1965 *342* 156—160

KREEK M J, F G GUGGENHEIM, J E ROSS and D F TAPLEY Glucuronide formation in the transport of testosterone and androstenedione by rat intestine *Biochim biophys Acta* (Amst) 1963 *74* 418—427

LEHTINEN, A, V NURMIKKO and K HARTIALA Duodenal glucuronide synthesis I Identification of estradiol glucuronide as a conjugation product of estradiol by the rat duodenal mucosa Qualitative studies *Acta chem scand* 1958 *12* 1585—1588

LEYBOLD, K and H J STAUDINGER Geschlechtsunterschiede im Steroidstoffwechsel von Rattenlebermikrosomen *Biochem Z* 1959 *331* 389—398

MOSEBACH K O W DIRSCHERL K RATH and G RIECK Die initialen Metabolite von Testosteron in den Zellfraktionen der Rattenleber und niere in vivo *Hoppe Seylers Z physiol Chem* 1966 *344* 197—208

…STEDT W and K HARTIALA Steroid Metabolism by the Canine Intestine I Qualitative Experiments with Progesterone *Scand J Gastroent* 1969 *4* 483—488

…MURA T and R SATO Function of cytochrome P-450 of microsomes *J biol Chem* 1964 *239* 2370—2379

SCHRIEFERS H W CREMER and M OTTO Sexualcharakteristika des Stoffwechsels von Testosteron in der perfundierten Rattenleber *Hoppe Seylers Z physiol Chem* 1967 *318* 183—193

SHOLITON L J R T MARNELL and E E WERK, Metabolism of testosterone 4 C^{14} by rat brain homogenates and subcellular fractions *Steroids* 1966 *8* 265—275

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From the Department of Pharmacology, University of Uppsala Sweden

Incorporation and Disappearance of Sulfate in Different Regions of Mouse and Rat Costal Cartilage in vivo and in vitro

By

Georg Herbai

Received 16 December 1969

Abstract

Herbai, G *Incorporation and disappearance of sulfate in different regions of mouse and rat costal cartilage in vivo and in vitro* Acta physiol scand 1970 79 541—551

A rapid and simple method for liquid scintillation counting of ^{35}S-sulfate in biological tissues is described Sulfate incorporation per mg dry weight into selected lateral medial and intermediate regions of ribs was studied using costal cartilage from rats and mice *In vitro* cartilage pieces embracing the osteochondral junction had 2—4 times larger incorporation rate than the remaining homogeneous parts of the ribs both if entire rib cages or stripped and diced cartilage were incubated After *in vivo* injection to rats and mice the incorporation rates of sulfate into the region of the osteochondral junction was 2 6 times that found in "resting" cartilage in rats [illegible]

"resting" cartilage The "resting [illegible] for sulfate and the osteochondr[illegible] showed a rapid sulfate disappe[illegible] weeks similar to that of "resting cartilage The necessity of careful selection of costal cartilage samples with respect to regional differences is emphasized

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Much biochemical work has been done on cartilage tissue taken from different animal species Costal cartilage from rats is frequently used since the cartilaginous part of the chest cage is a more plentiful source of cartilage than the epiphyseal area of the long bones

In most studies of sulfate incorporation costal cartilage has been assumed to be a metabolically homogeneous tissue and only two investigators (Klebanoff *et al* 1968 Yde 1968) have selected the cartilage pieces from a topographical point of view In a recent study using an autoradiographic technique (Herbai 1970) marked regional differences were shown in the incorporation pattern of ^{35}S sulfate into the cartilage of the rat and mouse rib cage In growing animals the osteochondral junction of ea

rib showed a markedly increased uptake of ^{35}S labeled sulfate as compared with the remaining parts of the rib. Since the autoradiographic technique only provides a qualitative picture of the distribution pattern the purpose of the present work was to analyze the magnitudes of the previously observed topographical differences in sulfate uptake. Furthermore the disappearance rate of *in vivo* injected ^{35}S sulfate was investigated in different regions of mouse costal cartilage and bone tissue.

Material and methods

Animals

Growing female Sprague Dawley rats weighing about 110 g and growing female NMRI mice of different ages were used in the study. All the animals were obtained from Anticimex AB, Norrviken, Sweden. They were housed in plastic cages and had free access to pelletted food (Anticimex nr 210 for rats and no 213 for mice) and tap water. The temperature of the room was kept at 25° C and a 12/12 day/night rhythm was maintained. All injections were given i.v. by the orbital plexus route as described by Pinkerton and Webber (1964).

Different types of experiments

Several kinds of experimental procedures were carried out and the sequences of different steps are summarized in a flow sheet in Table I.

Cartilage source and dissection

After killing the animals by ether their chest cages were dissected free in situ. An anatomically

en samples from ... from 3 animals

as pooled togethe

ection were used

the entire chest c

measurements from their vertebral to their sternal attachments. On account of the bent shape of the ribs the lengths were the measured with the aid of sewing thread.

Incubation procedure

The incubations were performed in stoppe ... Ringer

phosphate solution which was prepared ... al

(1957). Magnesium sulfate was changed ... olu

tion in order to eliminate carrier sulfate

glucose and 1 μC $^{35}S/ml$ and it was saturated with oxygen before use. The incubations were carried out in a Warburg apparatus at 37° C for 4 hrs. At the end of the incubation period the reaction was stopped by immersion of the flasks into a boiling water bath for 10 min. Thereafter the cartilage samples were rinsed with physiological saline and kept in 50 ml isotonic Na_2SO_4 solution for 24 hrs.

Control experiments were performed in order to study the inhibition of sulfate incorporation. Samples were preincubated for 2 hr in the standard medium containing 10^{-3} M(?) potassium cyanide but not $^{35}SO_4$ and then the isotope was added. In each series of experiments control studies were also made with rib cage samples which were heated in boiling water for 10 min prior to incubation.

Cleaning and stripping of the cartilage

For cleaning and stripping of the slender and brittle ribs (this was especially the case with mouse cartilage) from the firmly adherent muscle and connective tissue the whole cartilage block (Fig. 1) was placed into a 80° C water bath for 1 min. After this heating the ribs could be easily and thoroughly stripped with small forceps. All dissection and cutting work was carried out under a dissection microscope with 6 x magnification.

Processing of the samples for liquid scintillation counting

After measuring the dry weights the samples were directly placed into glass counting vials[1]

[1] From Packard Instr. Co. U.S.A.

TABLE I Sequence of experimental steps in 3 different kinds of experiments

In vitro alt I	*In vitro alt II*	*In vivo*
(use of stripped diced samples)	(use of uncleaned rib cage block)	Inject 3 μC $^{35}SO_4$ +3 μg Na_2SO_4 per g body weight
kill the animals by ether	kill the animal by ether	kill the animal by ether after 24 hrs
remove rib cage	remove rib cage	remove rib cage
cut out the block according to Fig 1	cut out the block according to Fig 1	cut out the block according to Fig 1
clean and strip cartilage rapidly without heating	incubate the block in Krebs-Ringer medium with 1 μC $^{35}SO_4$ per ml for 4 hrs →	dip the block into 80 C water for 1 min
dissect regions (Fig 1)	stop the reaction by boiling for 10 min	clean and strip cartilage
dice regions into 1—2 mm cylinders	rinse the sample in physiological saline	dissect regions (Fig 1)
pool diced regions from 3 rib cages	place the sample into isotonic Na_2SO_4 solution for 24 hrs →	dice regions into 1—2 mm cylinders
incubate pooled regions in Krebs Ringer medium with 1 μC $^{35}SO_4$ per ml for 4 hrs		
stop the reaction by boiling for 10 min		pool diced regions from 3 rib cages
rinse the diced sample in physiological saline		
place the sample into isotonic Na_2SO_4 solution for 24 hrs →		dry the sample in hot oven at 80° C overnight
		weigh the cartilage on microtorsion balance
		place the samples into counting vials and add 1.5 ml oxidation mixture
add 10 ml scintillator solution, refrigerate and count ^{35}S content of the sample	← oxidize and evaporate the sample in sand bath at 300° C and dissolve residue in 0.5 ml 0.1 N HCl	←

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Bray type (1960) was then added to the samples

Composition
1000 ml p dioxane (A R)

(POPOP)[1]

100 ml methanol (A R)

standardization was used for quench correction The calibration factor of the spectrometer was determined by means of absolutely standardized solutions obtained from the Radiochemical Centre, Amersham and all counting results referred to 100 % efficiency In the disappearance experiment corrections were made for decay All results were then expressed as DPM ^{35}S per mg dry cartilage

Results

Evaluation of the method

In order to obtain clean cartilage without remnants of surrounding tissue it was necessary to heat the block prior to the dissection After many experiments the most suitable way was found to be immersion of the entire chest cage into hot water at 80° C In order to exclude the possibility that intense heating influences the incorporated radioactivity, mouse rib cages with previously *in vivo* incorporated ^{35}S sulfate were incubated for different times varying between 15 sec and 30 min in 80° C water No loss of incorporated radioactivity was found in cartilage samples during heating up to 15 min At 30 min a moderate loss was observed It is thus evident that the contact with hot water cannot have influenced the incorporated counts of the cartilage during the one minute immersion used routinely

The influence of the fuming acid combustion on the sulfate content of the flasks was also investigated Identical recoveries of the radioactivity were found in all cartilage samples whether ^{35}S was added to the vials prior to or after the heat oxidation procedure

Furthermore it has been established that the presence of different amounts of cartilage in the counting vial did not influence the counting efficiency until the amount of tissue exceeded 60 mg

Table II shows that potassium cyanide decreased the sulfate incorporation to 38 % of the control levels while boiling completely abolished all incorporation Heated controls were run in each series of experiments with similar results

TABLE II The effect of KCN and of heating on the *in vitro* incorporation of ^{35}S sulfate into mouse costal cartilage The figures were obtained from pooled cartilage tissue of three animals

	dpm ^{35}S per mg dry tissue	%
control	46 900	100
heated for 10 min	19	0 04
KCN 10^{-3} moles/l	17 800	38

[1] From Packard Instr Co U S A

TABLE III Incorporation of ^{35}S-sulfate into the "active" lateral and the "resting" medial regions of mouse and rat costal cartilage after incubation with ^{35}S sulfate *in vitro* for 4 hrs In some experiments stripped and diced cartilage samples were used, in other experiments rib cage blocks with all soft tissue were incubated Cleaning in the last case was made after incubation

Animals	Cartilage condition at incubation	Anatomical location	DPM ^{35}S per mg dry cartilage	lateral / medial
3 rats	stripped and diced	lateral medial	89,000 40,250	2 20
3 rats	whole rib cage incubated	lateral medial	33,100 16 150	2 05
3 mice	stripped and diced	lateral medial	104,300 26,200	3 93
3 mice	whole rib cage incubated	lateral medial	49,000 14,400	3 40

Studies on rat and mouse rib cartilage

The sulfate incorporation values into the lateral and medial pieces of rat and mouse cartilage during different experimental conditions are summarized in Table III The table shows that throrough stripping and dicing of the cartilage pieces prior to incubation markedly increased the rate of sulfate uptake over those experiments in which the incubations were carried out with cartilage covered with soft tissue The lateral "active" parts of cartilage incorporated more than twice as much ^{35}S sulfate than the medial "resting" pieces did during the same time Table IV demonstrates that also *in vivo* the sulfate incorporation is much more intense in the lateral portion In this experiment regions taken from medial and intermediate positions were also compared and the results showed no differences in activity between them The lateral regions incorporated, however, more than twice as much as the remaining parts of the rib in rats and more than four times as much as the "resting" pieces in mice

The sulfate incorporation pattern of costal cartilage was also studied with respect to the cranio caudal location of the ribs In this experiment the entire cartilage segment of each rib was included from the sternal attachment to the bony part involving the osteochondral junction according to Fig 1 Table V shows that the total

TABLE IV Incorporation of ^{35}S sulfate into the "active" lateral and the "resting" medial and intermediate regions of mouse and rat costal cartilage after *in vivo* injection

Animals	Anatomical location	DPM ^{35}S per mg dry cartilage	lateral / medial
3 rats	lateral intermediate medial	92 000 35 400 37 500	2 55
3 mice	lateral intermediate medial	70 000 15 900 16 100	4 35

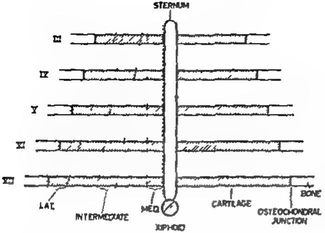

Fig 1 Schematic picture of the studied parts of the rib cage The dotted lines indicate how the cartilage was cut in order to separate and pool tissue pieces from different regions

amount of incorporated sulfate per rib increased in the caudal direction In DPM/mg dry cartilage, however, the sulfation activity was highest in the cranially located ribs

There is no evidence for any growth zone in the cartilage at the costo-sternal junction of the rib (Herbai 1970) and the main growth zone is located at the osteochondral junction This probably corresponds to the epiphyseal plate of long bones It is therefore of interest to measure the intensity of longitudinal growth of the bony and cartilaginous segments of the ribs The results of this experiment are seen in Fig 2 The rib lengths of mice were compared here from weaning to that age when the body weight ceased to increase The figure shows that the cartilaginous part of the rib grew but slightly and only at the start, while the bony segments of the ribs actively grew during the whole observed period Ossification is associated with a simultaneous destruction of cartilage and degradation of chondroitin sulfate This probably affects the disappearance of *in vivo* incorporated ^{35}S sulfate in certain regions of the rib cage In order to study the disappearance rate of sulfate in different costal regions the following experiment was made

15 female mice weighing about 22 g received a single i p dose of 20 $\mu C/g$ carrier free ^{35}S sulfate at the same time Three mice which represented the T_0 group for the

TABLE V Incorporation of *in vivo* injected ^{35}S sulfate into rib cartilage segments taken from different heights of mouse rib cages Each figure represents the mean of six separately dissected and studied ribs from a total of 3 animals Figures in brackets indicate range

Rib number	Total DPM ^{35}S per rib	Cartilage dry weight in mg	DPM ^{35}S/mg dry cartilage
III	586 (483 764	0.25 (0.2—0.3)	2387 (1937—2725)
IV	923 (827– 1160	0.6 (0.4—0.8)	1576 (1378—2125)
V	1268 (798—1850	0.93 (0.6—1.2)	1351 (1222—1542)
VI	16[illegible]0 (1375—1768	1.37 (1.2—1.6)	1219 (1098—1360)
VII	2221 1658 3016)	1.97 (1.4—2.4)	1130 (999—1254)

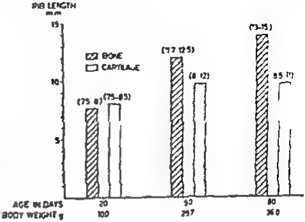

Fig 2 Lengths of the bone and cartilaginous parts of mouse ribs in growing animals of different ages Each column represents the mean length of 6 ribs (V, VI, VII) Figures in brackets indicate range Three animals at each age

sulfate disappearance were killed 24 hrs later and the subsequent groups of animals were sacrificed at weekly intervals up to 4 weeks After the usual preparation of the rib cages the following three segments were cut from each of ribs III—VII one including the osteochondral junction (3 mm bone + 3 mm cartilage) one a 6 mm long piece of dorsally adjacent bone and one a 6 mm long piece of ventrally adjacent 'resting cartilage Ten homologous pieces from each of three different animals were pooled and treated as described before (for further anatomical explanations see Fig 3) The results are summarized in Fig 3 and 4 Fig 3 shows schematically the regional localization of the investigated segments and the changes in the relations between the labeled part of the segment and the moving osteochondral junction during the observation period It can be seen that the initial labeling is continuously pushed dorsally on account of cartilage degradation and ossification The amounts of incorporated sulfate 24 hrs after the isotope injection are expressed in DPM/per mg dry tissue and these figures can be seen below the different uppermost segments in Fig 3 The bony segment incorporated only 1/7 of the resting cartilage value The osteochondral junction region consists of a mixture of relatively inactive bone resting cartilage and a highly active growth zone The presence of inactive bone

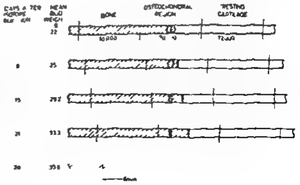

Fig 3 Schematic diagram of a rib at different intervals after a single ^{35}S injection to growing mice The figure shows progressing ossification disappearance of the label and longitudinal growth of the ribs The figures below the uppermost segments represent the amounts of incorporated ^{35}S in DPM/mg dry weight 24 hrs after the isotope injection

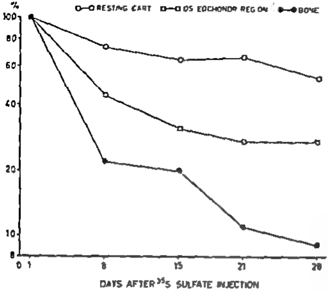

Fig 4 Disappearance of *in vivo* incorporated ^{35}S sulfate from different regions of the ribs of growing mice The ordinate shows the retained amount of ^{35}S in per cent of the initially incorporated value

in the region is the reason for the fact that the initially incorporated sulfate values on mg basis are so low In Fig 4 the percentual amounts of retained ^{35}S sulfate at ferent times are plotted on a semilogarithmic scale It can be seen that the sulfate content of the bony segment disappeared rapidly and only 9 % of the initial amount as found 4 weeks later The resting cartilage segments showed a very slow disappearance and more than the half of the initial sulfate content was still retained after 4 weeks The middle region which consists of 3 metabolically different segments showed a rapid sulfate disappearance during the first 2 weeks followed by a considerably slower disappearance at a rate similar to that of resting cartilage Moreover, it can be seen from the figure that at least the disappearance curves of the osteochondral junction and bony segment are non linear indicating the presence of several metabolic pools in these regions

Discussion

Several methods have been previously described by which $^{35}SO_4$ in different tissues could be rendered suitable for liquid scintillation counting Jeffay *et al* (1960) dissolved magnesium sulfate obtained by the hot acid oxidation procedure of Pirie (1932) using hot glycerol and dimethylformamide ethanol mixture The method is however, rather time-consuming and in our hands showed poor reproducibility Newton *et al* (1967 digested the tissues in acid and after several steps and operations suspended the radioactivity as a barium salt in thixotropic scintillator gel Several transfer operations and the gel barium salt homogenizing procedure makes this method complicated and laborious

The technique described in this study is rapid and simple but can be used only when the investigated quantity of tissue is below 30 mg All kinds of tissues (cartilage

bone blood or urine) can be processed in this way The procedure is well reproducible and gives complete recovery It requires few steps and avoids loss of rad activity through transfer operations Occasionally a weak precipitate can be seen in the solution after several hours at refrigerator temperature (+4° C) but it does not interfere with the counting It was found that the dioxane used in the scintillator solution should be crystalline at the storage temperature of +4 C Otherwise the counting efficiency was markedly reduced

In the *in vitro* experiments the practical principles described by Bostrom and Mansson (1953) were applied concerning the medium composition temperature incubation time metabolic and heat inhibition of the incorporation reaction The completely abolished incorporation of sulfate in the heated cartilage samples shows the effectivity of the rinsing procedure after incubation

As can be seen from Fig 1 a rather large part of the total cartilage mass of the chest cage was included in the study Since the osteochondral junctions showed a very high sulfate uptake activity in a previous autoradiographic study (Herbai 1970 a small piece of bone was included in each sample during the cutting of the ribs in order to ensure completeness of this highly active region which of course is not a homogeneous tissue anyhow

The regional pattern of incorporation was similar in rats and mice The *in vivo* distribution of sulfate corresponded well with the results of *in vitro* experiments It is evident from Table III that thorough cleaning stripping and dicing of the cartilage samples prior to incubation increased the tissue surface and its contact with the isotope resulting in accelerated sulfate incorporation The lack of differences in sulfate uptake between the medial and intermediate parts of the cartilage is in agreement with the results reported by Yde (1968) The relatively intense sulfate incorporation of the osteochondral junction seen in all present experiments corresponds well with the previously published autoradiographic results (Herbai 1970) Howell *et al* (1960) found twice as much sulphur in the proliferating cell zones than in resting cell tissue when they studied the chemical profile of calf costal cartilage slices This finding is in good agreement with the data reported here

Studying whole mouse rib cartilage segments it was found that the sulfate uptake per mg cartilage decreased in the caudal direction The activity pattern of each rib is composed of the highly active osteochondral junction and the relatively inactive resting cartilage segment In the caudal direction the length of the ribs and thus the proportion of the inactive part of the segment increases This could explain the diminishing activity per mg It is not known however if the sulfate uptake activities in the osteochondral junctions of different ribs are similar

It is evident from Fig 2 that the lengths of the cartilaginous segments did not change much while the bony part of the rib grew continually

The intense cartilage synthesis of the osteochondral junction is thus rapidly followed by a progressive ossification The degradation of chondroitin sulfate in rat costal cartilage has been studied by several authors Bostrom (1952) found the biological half life of previously injected sulfate to be 17 days in costal cartilage of

rats. Furthermore, Gross *et al.* (1960) while studying the turnover of chondroitin sulfate—protein complex in rib cartilage of adult rats, found a non linear disappearance rate of ^{14}C and ^{35}S from the complex when the data were plotted on a semilogarithmic scale. In neither of the studies the way of dissection was stated. The results presented here were obtained in animals during intense growth, a circumstance which makes their cartilage metabolism complex. In addition to a basal cartilage turnover which can be seen in adult animals there is also a net growth both in the circular and the longitudinal direction. This is the cause of the profound regional difference in the sulfate disappearance rates. The steady and slow disappearance of sulfate in the medial "resting" cartilage segments which do not grow longitudinally correspond with the results achieved by Bostrom in adult rats (Bostrom 1952). The segment which only contained bone incorporated a small amount of sulfate (compared to cartilage) and this label which probably represented the circular thickening of the bone disappeared very rapidly from the tissue. The middle region consists of a combination of perhaps 40 per cent inactive bone, 40 per cent moderately *active 'resting' cartilage and about 10 per cent highly active growth zone*. The slope of the first part of the sulfate disappearance curve makes it probable that the activity of the growth zone largely disappeared during the first two weeks after which this segment behaved as 'resting' cartilage. The bony part of the segment presumably contains so little activity that its influence is not discernible. If the slope during the last two weeks is extrapolated to zero time, the intercept is around 40 per cent which is of the same order of magnitude as the proportion of "resting" cartilage in the segment. The rapid disappearance of sulfate from the growth region is in good agreement with the findings of Weatherell and Weidmann (1963) who found a precipitous fall in bound sulfate in connection with replacement of cartilage by osseous tissue in femurs of foetal calves.

The heterogeneity of costal cartilage demonstrated above makes it evident that consideration must be paid to the anatomical origin of cartilage when tissue pieces are selected for studies, especially if growing animals are used.

I wish to express my thanks to Professor E. Bárány, for his stimulating help and criticism and to Mrs. B. Jansson for skilful technical assistance.
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References

[illegible] of chondroitin sulfuric acid. *J. biol. Chem.* [illegible]
[illegible] ate group of the [illegible] 57
[illegible] ions in a liquid [illegible]
[illegible] otein complexes [illegible]
[illegible] strogen sensitive [illegible]
growth zones in rat and mouse cartilage. *Acta physiol. scand.* [illegible]—358

HOWELL, D. S., E. DELCHAMPS, W. RIEMER and T. KIEM: A profile of electrolytes in the cartilaginous plate of growing ribs. *J. clin. Invest.* 1960, 39, 919—929.
JEFFAY, H., F. O. OLUBAJO and W. R. JEWELL: Determination of radioactive sulfur in biological materials. *Anal. Chemistry* 1960, 32, 306—308.
KLEBANOFF, S. J., D. D. DZIEWIATKOWSKI and G. J. OKINAKA: The effect of ascorbic acid oxidation on the incorporation of sulfate by slices of calf costal cartilage. *J. gen. Physiol.* 1958, 42, 303—317.
NEWTON, W. R., J. MURPHY and L. E. MULLINS: Determination of radiosulfate in biologic fluids and tissues. *J. Lab. clin. Med.* 1967, 69, 518—521.
PINKERTON, W. and M. WEBBER: A method of injecting small laboratory animals by the opthalmatic plexus route. *Proc. Soc. exp. Biol. (N. Y.)* 1964, 116, 959—961.
Pr [illegible] ism of methionine [illegible]
U [illegible] 1957 pp. 149—
W [illegible] bound sulphate in [illegible]
YDE, H.: A simplified technique for the determination of growth hormone dependent sulfation factor using intact animals. *Acta endocr. (Kbh.)* 1968, 57, 557—564.

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Dilatation of a Medium-Sized Artery Immediately after Local Changes of Blood Pressure and Flow as Measured by Ultrasonic Technique

By

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Abstract

INGEBRIGTSEN, R and S LERAAND *Dilatation of a medium-sized artery immediately after local changes of blood pressure and flow as measured by ultrasonic technique* Acta physiol scand 1970 79 552—558

Th[illegible] artery has [illegible] s. On [illegible] crease [illegible] ement arterial diameter—by which it was possible to measure changes in the diameter in the order of 0 001 mm—we found an immediate increase, in the order of 10 %, in the diameter of the femoral artery on the opening of the a v fistula This increase remained as long as the fistula was patent The results were identical in 5 dogs

The effect on the diameter of clamping the artery for some time with consequent removal of the clamp was examined in 5 dogs Removal of the clamp itself resulted in only a small and temporary increase in the diameter of the artery It is concluded that the dilatation of the artery on opening of the fistula must be connected to the sudden increase in flow

The dilatation of the artery proximal to an arterio-venous fistula was described about 200 years ago by W Hunter The intimate mechanism behind this dilatation has since been a puzzle to physiologists In a previous study of the circulation in and around the fistula we have found and measured a large drop in pressure—as much as 50—70 mm Hg—along 6—7 cm of the artery proximal to the fisula (Ingebrigtsen and Wehn 1960) Blood flow increased enormously when the fistula was opened by about 1000 % or even more depending on the size of the fistula (Ingebrigtsen *et al* 1962)

In the present studies of the mechanism of the dilatation of the artery proximal to an a v fistula we have been able to measure the dilatation from its very beginning by means of a technique with ultrasonic transmission With this method it is possible to record changes in the diameter of a vessel in the order of 0 001 mm The velocity of ultrasound in blood and tissue is nearly constant and is approximately 1500 m/sec

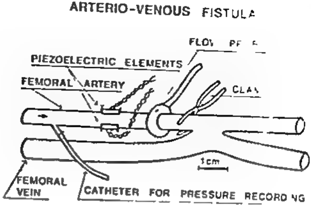

Fig 1 Schematic drawing of application of instruments on the femoral artery

Material and methods

The experiments were ... exposed below and abov... to the exit of the saph... (no 7—0) after mak... recorded by Statham p... transducers and a Sanborn 4-channel direct writing recorder Flow was measured by an electromagnetic square wave flowmeter ("Nycotron Oslo Norway) Anesthesia was induced by i v injection of Nembutal 25 mg/kg For pressure recording a catheter was introduced through a branch of the artery 1 5—2 cm above the site of the piezoelectric elements from which a continuous recording of variations of the diameter was obtained (Fig 1)

Two small piezo-electric elements (lead zirconate titanate) were sewn to the arterial wall exactly diametrically opposite to each other One of the elements was used as a transmitter and the other as a receiver of ultrasonic pulses The transit time which is proportional to the diameter was measured continuously and recorded with a Sanborn recorder In order to make certain that our recordings of increases in arterial diameter reflected a real dilatation of the artery additional experiments were carried out in two dogs. Here we used two pairs of piezoelectric elements each pair being applied to the artery perpendicularly on each other with a distance between them of 10 mm The values for the two recorded diameters ran absolutely parallel to each other (Fig 2)

In order to examine the possible effect of changes in temperature and of changes due to clamping of the artery distal to the position of the elements (and such clamping is an inevitable procedure in the establishment of the fistula) we placed the clamp on the artery 3 cm distal to the pressure recording catheter during experiments The alterations in diameter subsequent to the release of the clamp were then recorded

Results

In order to establish the a v fistula the femoral artery had to be clamped for about 1/2—3/4 hr In 5 consecutive and successful experiments the results of *clamping of the femoral artery* for 10 and 60 min—followed by removal of the clamp—were about the same On removal of the clamp the blood pressure—measured a couple of cm above the site of the elements—dropped immediately by 5—8 mm Hg and then remained stable at this new level The diameter of the femoral artery showed an immediate decrease of about 0 05 mm It then rose to 0 1 mm above the original level remained at this level for a few seconds whereafter it slowly returned to its original value in the course of 3 min (Fig 3 exp no 2)

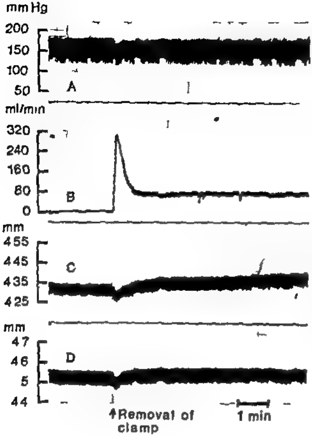

Fig 2

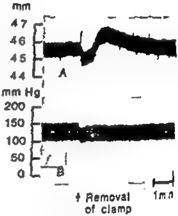

Fig 3

Fig 2 Exp no 5 Simultaneous measurements of arterial diameter with two pairs of piezo-electric elements The normal femoral artery has been clamped for one hour A Blood pressure in the femoral artery B Flow in the femoral artery C & D Diameter of the femoral artery above the clamp measured by two independent pairs of piezo-electric elements (see text)

Fig 3 Exp no 2 The effect of clamping and reopening of a femoral artery on arterial diameter A normal femoral artery has been clamped for 20 min A Diameter of the femoral artery B Blood pressure in the femoral artery

On the *opening of the fistula* by removal of the arterial clamp local blood pressure immediately dropped by about 10—15 mm Hg and then stayed at this level In the experiment of Fig 4 it then increased a few mm Hg in the course of 9 min On approaching the fistula an increasing fall in pressure was recorded After a small decrease lasting 4 sec the diameter of the femoral artery increased rapidly by 0 3—0 5 mm and did not change later as long as the fistula was patent (Fig 4 and 5)

In exp no 1, flow in the femoral artery measured about 1 1/2 cm distal to the site of the elements and also proximal to the elements on the external iliac artery reached levels of about 900 ml min Fig 6)

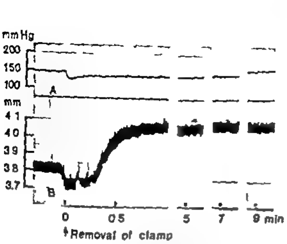

Fig 4

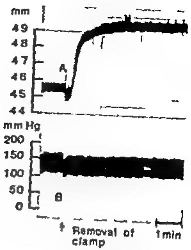

Fig 5

Fig 4 The effect on arterial diameter of opening up of an a v fistula by removal of arterial clamp (Exp no 1) A Blood pressure in the femoral artery B Diameter of the femoral artery (see Fig 1)

Fig 5 The effect on arterial diameter of opening up of an a v fistula by removal of arterial clamp (Exp no 2) A Diameter of the femoral artery B Blood pressure in the femoral artery (see Fig 1)

The test of clamping and unclamping the artery was repeated in the same animal after an interval of 20 min In the experiment of Fig 6 clamping of the femoral artery reduced its diameter slowly from 4 55 mm to 4 35 mm in the course of 8 min On removal of the clamp thereby opening the fistula local blood pressure dropped by 15—20 mm Hg Flow rose from 0 to 700 ml/min The diameter of the femoral artery showed a small immediate reduction lasting only a few seconds and then increased from 4 35 mm to 4 50 mm a level at which it remained for at least 20 min In another dog the increase of the diameter on the opening of the fistula was from 4 85 to 5 0 mm

On unclamping the artery and reopening the fistula in such a second test the dilatation of the artery was apparently somewhat less marked than on the first opening of the fistula

Discussion

Clamping the femoral artery may have various effects the temperature of the arteri wall may be influenced the trauma of the clamp and the reduction of flow to zer may affect the nutrition of the arterial wall We have therefore examined the effec of applying a clamp on the normal femoral artery The blood pressure rose by 5— mm Hg above the clamp and dropped immediately to the same d removal

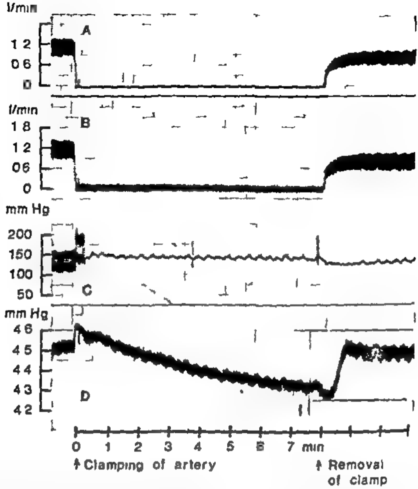

Fig 8 The effect on arterial diameter and flow of a second clamping and reopening of the femoral artery with opening of the a v fistula (Exp no 1) A Flow in the femoral artery B Flow in the external iliac artery C Blood pressure in the femoral artery D Diameter of the femoral artery (see Fig 1)

The effect of the clamping on the *diameter of the artery* showed small variations and was independent of the length of time of the clamping The small increase in diameter on release of the clamp was about the same—and even less—after clamping for 1 hr as after 10 or 20 min (Fig 2 and 3)

The effect on the diameter of the artery of returning to normal blood flow gave a diameter response quite different from that seen on opening of the arterio-venous fistula After a small reduction of the diameter lasting about 10 sec it increased for 15 sec by a maximum of 0 1 mm in relation to its original size This increase was temporary the diameter returning to the original level in the course of 3 min (Fig 3) The dilatation resulting from the opening of the arterio venous fistula on the

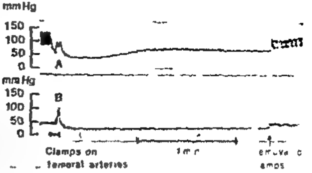

Fig 7 Blood pressure measurements in the anterior tibial artery of both calves A Blood pressure in the right normal tibial artery B Blood pressure in the left calf, in which an established a v fistula had been open for 15 min

other hand, amounted to as much as 10 % of the original diameter and lasted as long as the fistula was open

It is reasonable, therefore, to regard the clamping itself as a negligible factor as regards the development of arterial dilatation immediately after opening of the fistula This dilatation occurred immediately on the opening of the fistula coincident with a local fall of blood pressure and an enormous increase in flow

We have also tried to repeat the experiments of d Silva and Fouche (1960) These authors reported a quickly occurring dilatation of the femoral artery in cats after the establishment of a functional shunt established by means of a tube between the tibial artery and the femoral vein of the other leg On turning a tap in the tube the dilatation of the femoral artery was observed by angioradiography d Silva and Fouche have not reported the full details of their experiments In similar experiments on dogs, using angiography, we have been unable to observe such an immediate arterial dilatation after opening of a functional shunt of this type

In this connection, we want to mention the experiments of Schretzenmayr (1933), Fleisch (1935) and Hilton (1959) Schretzenmayr and Hilton worked on the femoral artery of cats, Fleisch on the same artery of dogs They all found dilatation of the femoral artery subsequent to peripheral vascular dilatation caused by muscular activity or injection of vasodilating substances The dilatation of the femoral artery was not observed in all the experiments In conclusion the authors agreed to the existence of a dilating mechanism originating in the periphery and ascending in the wall of the femoral artery

The mechanism of the dilatation of the femoral artery is obviously a different one in our experiments in which the effect of the arterio-venous fistula during the first minute after its opening is not distal hyperemia but ischemia In one dog we have recorded the blood pressure in the anterior tibial artery of both calves before and after opening of the fistula Flow was found to be 300 ml/min in the fistulous femoral artery and 25 ml/min in the normal right femoral artery The blood pressure in the left, fistulous artery after opening of the fistula was 40 mm Hg and in the normal artery 100 mm Hg On clamping both femoral arteries above the fistu sure in the fistulous tibial artery dropped from 40 mm to 25 mm

that level for at least 2 min, whereas in the normal tibial artery it dropped from 100 mm to 35 mm Hg, and from this level rose slowly to 70 mm Hg in the course of 1 min (Fig 7) This gives an idea of the poor circulation distal to an a-v fistula on the femoral vessels in the first minutes after its establishment

Another prominent feature of our experiments is the finding that the dilatation of the femoral artery lasts as long as the fistula is open

In trying to explain the dilatation occurring in our experiments we have excluded an effect resulting from the clamping of the artery One primary effect of opening of the fistula is the drop of local blood pressure This is immediately followed by an enormous increase in blood flow Between the opening of the fistula and the increase of the diameter there is in all experiments, a time lag of 15—20 sec in which the diameter decreases by about 0 1 mm This is a usual effect of a drop in blood pressure The following increase of the diameter, which is illustrated in Fig 4, is accompanied by only a very small increase in blood pressure

We therefore believe that the lowering of the local blood pressure which follows the opening of the fistula may be excluded as a cause *per se* for the dilatation of the artery With our present knowledge, the only parameter which may be imagined as causing the described dilatation of the artery is the enormous increase in blood flow

References

[illegible]ch A Les reflexes nutritifs ascendants producteurs de dilatation arterielle *Arch int Physiol* 1935 *41* 141—167

[illegible]ton S M A peripheral arterial conducting mechanism underlying dilatation of the femoral artery and concerned in functional vasodilatation in skeletal muscle *J Physiol* (Lond) 1959 *149* 93—111

Hunter W Further observations upon a particular species of aneurysm *Med Obs Soc Phys* (Lond) 1762 *II* 390

Ingebrigtsen R and P Wehn Local blood pressure and direction of flow in experimental arterio-venous fistulae *Acta chir scand* 1960 *120* 142—150

Ingebrigtsen R J Krog and S Leraand, Velocity and flow of blood in the femoral artery proximal to an experimental arterio venous fistula *Acta chir scand* 1962 *124* 45—53

Schretzenmayr A Ueber Kreislaufregulatorische Vorgange an den grossen Arterien bei der Muskelarbeit Pflugers *Arch ges Physiol* 1933 *232* 743—748

D Silva J L and R F Folche The effect of changes in blood flow on the calibre of large arteries J Physiol 1960 (Lond) *150* 23P—24P

Acta physiol. scand. 1970. 79. 559—563

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Effects of Secretin on Fat Mobilizing Lipolysis and Cyclic AMP Levels in Rat Adipose Tissue

By

R W Butcher and L A Carlson

Received 22 December 1969

Abstract

Butcher, R W and L A Carlson *Effects of secretin on fat mobilizing lipolysis and cyclic AMP levels in rat adipose tissue* Acta physiol scand 1970 79 559—563

Secretin, 0 0025 µg/ml, stimulated fat mobilizing lipolysis in rat adipose tissue in vitro while cholecystokinin was without eff
14, 15—27, 1—23 and 1—6
concentration of cyclic AMP
M Secretin also increased th
fat cells Nicotinic acid and
lipolytic rate and accumulation of cyclic AMP

A variety of hormones stimulate the rate of fat mobilizing lipolysis of adipose tissue triglycerides In the rat these include the catecholamines growth hormone, ATCH glucagon, TSH, and ACTH (Rudman 1965, Carlson 1968), and recently secretin was found to have lipolytic activity at low concentrations (Carlson 1968 Rudman and Del Rio 1969)

All of the hormones listed above appeared to increase the rate of lipolysis by increasing intracellular concentrations of cyclic AMP (Butcher *et al* 1968), which then activates the rate limiting enzyme in the fat mobilizing lipolytic pathway the hormone sensitive triglyceride lipase This being the case it was of interest to see if secretin also activated lipolysis through cyclic AMP In addition Drs Jorpes and Mutt who kindly provided the purified secretin used in this work also made available fragments of secretin, and the effects of these fragments on lipolysis have been studied in the hope of gaining some information about the site at which secretin interacts with the fat cell

Experimental Procedures

Measurements of lipolysis and cyclic AMP

Adipose tissue of fed rats (180—200 g) was incubated in Krebs-Ringer bicarbonate 2 % albumin and 0 1 % glucose as described in detail previously (Bizzi and C
The lipolytic rate was estimated from determination of the amount of glycerol

that level for at least 2 min whereas in the normal tibial artery it dropped from 100 mm to 35 mm Hg and from this level rose slowly to 70 mm Hg in the course of 1 min (Fig 7) This gives an idea of the poor circulation distal to an a v fistula on the femoral vessels in the first minutes after its establishment

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References

[illegible] A Les reflexes nutritifs ascendants producteurs de dilatation arterielle *Arch int Physiol* 1935 *41* 141—16

[illegible]son S M A peripheral arterial conducting mechanism underlying dilatation of the femoral artery and concerned in functional vasodilatation in skeletal muscle *J Physiol* (Lond) 1939 *149* 93—111

HUNTER W Further observations upon a particular species of aneurysm *Med Obs Soc Phys* (Lond) 1762 *II* 390

INGEBRIGTSEN R and P WEHN Local blood pressure and direction of flow in experimental arterio venous fistulae *Acta chir scand* 1960 *120* 142—150

INGEBRIGTSEN R J KROG and S LERAAND Velocity and flow of blood in the femoral artery proximal to an experimental arterio venous fistula *Acta chir scand* 1962 *124* 45—53

SCHRETZENMAYR A Ueber Kreislaufregulatorische Vorgange an den grossen Arterien bei der Muskelarbeit Pflugers *Arch ges Physiol* 1933 *232* 743—748

D SILVA J L and R F FOUCHE The effect of changes in blood flow on the calibre of large arteries J Physiol 1960 (Lond) *150* 23P—24P

Discussion

Secretin is a potent stimulator of lipolysis in isolated rat fat in contrast to cholecystokinin The stimulation appears to require an intact or almost intact peptide molecule Similar observations have been reported by Rudman and Del Rio (1969) The antilipolytic agents nicotinic acid and PGE_1 were effective also against the secretin induced stimulation of lipolysis which effect once again can be correlated to interaction with the levels of cyclic AMP in the tissues The physiologic implication of the lipolysis stimulating effect of secretin is not known It is however of interest that this hormone has hormonal effects outside the sphere of the digestive apparatus *i e* unrelated to stimulation of the exocrine or endocrine parts of pancreas

Secretin activated adenyl cyclase in cell free preparations where possible effects of the hormone on the phosphodiesterase were minimized In addition, the relative homogeneity of the isolated fat cell preparation makes the possibility of displacements or the intervention of local hormone release seem unlikely Finally the synergism between methylxanthines and secretin on cyclic AMP levels in isolated fat cells is very difficult to explain except by the activation of the adenyl cyclase system by secretin Thus, it would appear that secretin like so many other hormones activates the adenyl cyclase system of adipose tissue

The ability of PGE_1 insulin and nicotinic acid to antagonize the effects of secretin were not unexpected since these agents also antagonize the other lipolytic hormones

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References

BIZZI A and L A CARLSON Stimulatory effect of ethanol on glycerol release from rat adipose tissue *in vitro* *Life Sci* 1965 4 2123

BUTCHER R W R J HO H C MENG and E W SUTHERLAND Adenosine 3 5 monophosphate in biological materials II The measurement of adenosine 3 5 monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine *J biol Chem* 1965 240 4515

BUTCHER R W and C E BAIRD Effects of prostaglandins on cyclic AMP levels in fat and other tissues *J biol Chem* 1968 243 1713

BUTCHER R W C E BAIRD and E W SUTHERLAND Effects of lipolytic and antilipolytic substances on adenosine 3 5 monophosphate levels in isolated fat cells *J biol Chem* 1968 243 1705

CARLSON L A Lipolysis of adipose tissue triglycerides Regulation Physiological and Clinical Significance in Protein and Polypeptide Hormones M Margoulies Ed *Excerpta med* (Amst Sect I 143—149

RODBELL M Metabolism of isolated fat cells I Effects of hormones on glucose metabolism and lipolysis *J biol Chem* 1964 239 375

RUDMAN D M DIGIROLAMO M F MALKIN and L A GARCIA In *Handbook of Physiology* Section 5 Adipose Tissue A E Renold and G F Cahill Jr Eds American Physiological Society Washington pages 533—540

RUDMAN D and A E DEL RIO Lipolytic effect of a peptide fragment of porcine secretin *Endocrinology* 1969 85 610

VAUGHAN M and D STEINBERG Effect of hormones on lipolysis and esterification of free fatty acids during incubation of adipose tissue *in vitro* *J Lipid Res* 1963 4 193

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Effects of Vasoactive Drugs on Circulation in Canine Subcutaneous Adipose Tissue

By

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Abstract

FREDHOLM B B B ÖBERG and S ROSELL *Effects of vasoactive drugs on circulation in canine subcutaneous adipose tissue* Acta physiol scand 1970 79 564—574

The circulatory effects of acetylcholine bradykinin isoprenaline histamine 5 hydroxytrypt
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i induced a moderate blood flow increase with no change or a decrease of tissue volume
cat ng a constriction of the venous section The vascular responses to noradrenal ne infus ons
re essentially similar to those produced by sympathetic nerve activation *i e* constriction of arterioles and veins and a markedly increased capillary filtration coefficient (CFC) Histamine and bradykinin induced increases of CFC of the same magnitude as did sympathetic nerve stimulat on wl ereas acetylcholine isoprenaline 5 HT and PGE_1 induced smaller increases of CFC Sympathetic nerve stimulation superimposed upon infus ons of the latter drugs produced a further marked increase of CFC while nerve stimulation applied during a period of histam ne or bradykinin infus on produced no further rise or even a decrease of CFC It is discussed that the pronounced CFC increase during sympathetic nerve stimulation is due to an increased permeability of the capillary membrane

Studies of the blood circulation in adipose tissue and its regulation by nervous and humoral factors seem to be of importance for many reasons Adipose tissue represents a relatively large tissue mass demanding a substantial portion of the cardiac output Regional vasomotor adjustments in this vascular bed must therefore influence the overall hemodynamics Furthermore energy rich material in the form of free fatty acids (FFA) is mobilized from adipose tissue and this process is dependent on an adequate blood supply

In previous studies the sympathetic vasomotor fibre control of circulation in subcutaneous adipose tissue in dogs was examined (Ngai Rosell and Wallenberg 1966 Öberg and Rosell 1967) Activation of the regional sympathetic fibres was shown to produce a constriction of resistance and capacitance vessels a net transcapillary absorption of tissue fluid and an increased hydrodynamic conductivity of the capillary

membrane, as demonstrated by an elevated capillary filtration coefficient (CFC) during the stimulation periods

In the present series of experiments, the characteristics of subcutaneous adipose tissue circulation have been further analyzed by studying the vascular responses to vasoactive drugs The adjustments of the various functionally differentiated series-coupled sections of the vascular bed, were followed by means of a plethysmographic technique CFC was repeatedly determined during infusion of drugs and the values compared with those obtained with stimulation of the regional vasoconstrictor fibres and those found when stimulation and drug infusion were combined Such comparisons were expected to provide information concerning the possible mechanisms responsible for the increase of CFC during sympathetic stimulation

Methods

Experiments were performed on 24 female mongrel dogs anesthetized with sodium pentobarbital, Nembutal 30 mg/kg Additional doses were given if necessary during the course of the

the fat as possible The artery was cannulated and the vascular bed perfused through an exteriorized loop from the right femoral artery A drop recorder operating an ordinate writer

animal via a funnel connected to the left femoral vein The preparation was then placed in a plethysmograph which was filled with body warm Tyrode solution and connected to a piston recorder for continuous measurement of tissue volume changes The cannulated vessels and the nerve passed through a close fitting opening in the plethysmograph which was made water tight with a water repellent grease (Plastibase Squibb) —The cut end of the nerve was placed on a bipolar silver electrode for electrical stimulation The stimuli were delivered from a Grass stimulator

Blood pressure and flow were recorded on a Grass polygraph recorder tissue volume changes on a smoked drum

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For further details concerning the CFC determina
1963) Folkow *et al* (1963)
present study Acetylcholine Bradykinin Histamine
rtrate (5 HT) Mepyramine maleate Isoprenaline
andin E_1 (PGE_1 Upjohn Co) The doses of the
drugs are expressed as the amount of active substance All the drugs were dissolved in isotonic saline immediately before administration Infusion rates were 0 1—0 4 ml/min

Results

The responses of the different series coupled vascular sections in the adipose tissue to the various vasoactive drugs were analyzed in 15 animals The responses of capillary resistance vessels were obtained from changes in flow resistance A

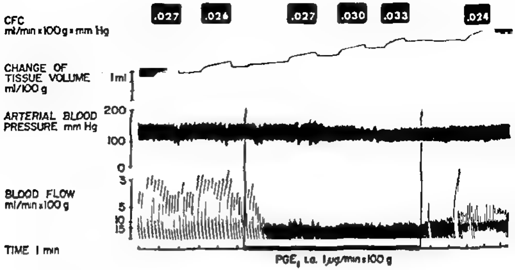

Fig 1 Effects of intraarterial infusion of prostaglandin (PGE1) on blood flow, tissue volume and CFC of an isolated subcutaneous adipose tissue preparations (weight 32 g)

of the capacitance vessels (veins) were followed in terms of rapid phasic changes in tissue volume and alterations in the capillary function was assessed by repeated determinations of the capillary filtration coefficient (CFC)

Resting values for blood flow and CFC i e the values obtained prior to drug administration or nerve stimulation averaged 9 5 ml/100 g/min (range of variation 3 7—18 ml/100 g/min) and 0 023 ml/100 g/min/mm Hg (0 0016—0 038) respectively These values agree with those found earlier (Öberg and Rosell 1967) The highest values for blood flow and CFC were found in the smallest tissue preparations (with tissue weights of about 20 g as compared with the average tissue weight of 35 g for the whole series)

Prostaglandin E_1 Fig 1 illustrates recordings from one experiment where prostaglandin E_1 (PGE_1) was administered as a constant arterial infusion Blood flow increased despite an unchanged perfusion pressure indicating a relaxation of the resistance vessels i e the arterioles where the major part of the resistance function resides The volume recording shows a rapid but moderate volume gain when the infusion starts indicating an increased regional blood content presumably due to dilatation of the veins

Following the initial rapid volume increase there is a further slow but constant increment of tissue volume interpreted as a net transcapillary fluid filtration due to a capillary pressure rise following the dilatation of precapillary resistance vessels CFC determined at intervals shows an increase by approximately 20 per cent during drug infusion

From a qualitative point of view a similar response pattern as demonstrated in Fig 1, was obtained in all experiments with PGE_1 although there were quantitative dif

TABLE I

Drug	Dose stim freq M/cps	Number of exp	Control	
			Flow ml 100 g min	CFC ml 100 g min/ /mmHg
Histamine	3×10^{-7} [1] 4×10^{-8} [2]	11	9 9 (3 1—16)	024 (016—036
PGE_1	$1—5 \times 10^{-9}$	5	9 4 (5 9—14 5)	025 (020—032)
	$1—6 \times 10^{-9}$	[illegible]	6 7 (4 5—9)	024 (019—027)
	$1—9 \times 10^{-7}$	11	4 8 (1 9—9 8)	021 (019—026)
	$1—2 \times 10^{-6}$	4	3 7 (3 5—4 0)	025 (0 20—036)
Isoprenaline	1×10^{-7} [1] 5×10^{-8} [2]	11	4 7 (2 1—7 5)	023 (020—030)
Acetylcholine	$10^{-6}—10^{-9}$	4	6 4 (4 4—7 5)	023 (015—036)
Bradykinin	$3—5 \times 10^{-10}$	3	10 4 (8 7—11 4)	017 (017—018)
5—HT	$1—5 \times 10^{-6}$	5	17 2 (11 5—23)	037 (022—048)
Noradrenaline	$10^{-6}—10^{-7}$	6	9 2 (1 9—12)	023 (018—031)
Symp Stim	3—8	27	7 5 (3 1—18)	022 (016—032)

The Values given are the mean responses Figures within parenthesis denote range of variation — —[1] Initial maximal response —[2] Steady state response

ferences dependent upon other factors *e g* the dose used the prevalent vascular tone prior to drug administration etc In Table I data from the various experiments have been collected and grouped according to the drug and calculated concentration of the agent in blood It shows that a clearcut vasodilatation was obtained with such low blood concentrations of PGE_1 as $1—5 \times 10^{-9}$ M Such low concentrations of PGE_1 also produced a dilatation of the veins and an increase of CFC It therefore seems as if the vascular smooth muscle elements in adipose tissue are extremely sensitive to PGE_1

Acetylcholine and isoprenaline These drugs produced qualitatively the same type of responses as those illustrated in Fig 1 Acetylcholine even in huge doses (blood concentrations of 10^{-3} M) did not induce blood flow values of the same order of magnitude as was obtained by much smaller doses of PGE_1 Also the capacitance vessel response and the shifts in CFC appeared to be less pronounced with acetylcholine This drug therefore seems to be a relatively poor dilator substance as far as adipose tissue circulation is concerned

Isoprenaline on the other hand produced a high blood flow and CFC comparable to those obtained with PGE_1 but the venous responses were less pronounced

	With drug/stimulation/			
Flow ml/100 g/min	% change	CFC ml/100 g/min/mmHg	% change	Reg blood vol % change
22 5 (5 2—45)	+113 (22—195)	044 (025— 060)	82 (25—154)	+6 4 (2—15)
13 0 (7 5—22)	+ 35 (0—69)	029 (020— 040)	16 (0—28)	+4 3 (0—8 5)
13 3 (7 5—22)	+100 (62—180)	028 (019— 038)	13 (0—40)	+5 5 (3—10)
10 5 (4 7—19 2)	+127 (30—235)	027 (020— 033)	27 (11—37)	+8 3 (3—17)
11 8 (9 5—14 0)	+210 (165—290)	030 (025— 040)	20 (11—25)	+7 4 (4—10)
12 2 (3 2—24)	+151 45—364	027 (021— 033)	15 (0—40)	+3 (1—5)
12 5 (7 5—16)	107 (12—240)	028 (018— 040)	20 (11—32)	+4 (0—8)
35 3 (28—42)	246 150—380	037 (031— 044)	114 (82—165)	+13 (9—19)
(29 7[1]—21 1[1] (12 5—27)	23 9—32	040 (022— 052)	9 (0—15)	—3 (0—8)
3 (0 6—6 2)	— 68 (30—85)	037 (033— 038)	63 (25—100)	—14 (5—25)
2 3 (1 0—5 5)	— 66 (27—85)	038 (026— 055)	73 (33—130)	—14 (6—40)

Histamine and bradykinin Infusions of histamine and bradykinin also caused a dilatation of resistance and capacitance vessels in adipose tissue Both drugs seemed to be efficient vasodilators Maximal blood flows of approximately 35 -45 ml/100 g/min, were produced

The above mentioned drugs usually induced pronounced increases of CFC In comparison with PGE_1 isoprenaline and acetylcholine CFC increased usually two to four times more for a given reduction in flow resistance when bradykinin or histamine were administered This is clearly demonstrated in Fig 2 Changes in CFC produced by various procedures are plotted here against the simultaneously induced alterations of flow resistance From such comparisons it seems reasonable to suggest that the pronounced increase of the CFC following bradykinin and histamine administration is different from that produced by PGE_1 acetylcholine and isoprenaline

Comparisons between PGE_1 acetylcholine and histamine To further test the sensitivity of the adipose tissue vascular bed to various dilator drugs, a series of 9 expts were performed where the effects of increasing concentrations of PGE_1 acetylcholine and histamine, given as i a injections on blood flow were studied The results from these experiments are shown in Fig 3 It can be seen that on a molar basis, PGE_1

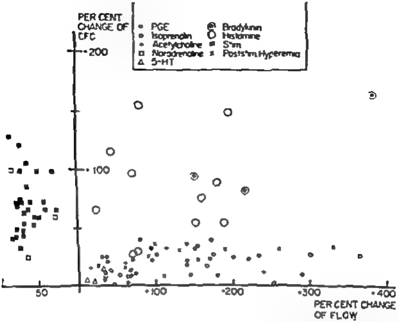

Fig 2 Relation between per cent change in blood flow and in CFC in subcutaneous adipose tissue, produced by administration of various vasoactive drugs and by regional vasomotor fibre stimulation.

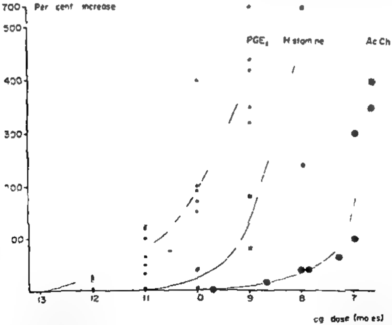

Fig 3 Comparison of the magnitude of vasodilatation (expressed as per cent increase of blood flow from control) produced by close intraarterial injections of acetylcholine, histamine PGE₁ The doses are given as moles of active substance Each point represents one in

is the most effective dilator of the adipose tissue vascular bed being roughly 1000 times more potent than acetylcholine and 10 times as potent as histamine

5 hydroxytryptamine Administration of 5 HT to the *adipose tissue vascular bed* resulted in an initial moderate relaxation of the resistance vessels This vasodilatation was however not well maintained and the blood flow returned more or less completely to control levels despite continued infusion There were no indications from the volume recording of an increased tissue volume during 5 HT infusion The volume either remained unchanged or even decreased in three out of five expts despite the dilatation of precapillary vessels and the resulting pressure rise in the venous compartment This finding indicates that 5 HT produced a constriction of the veins although admittedly rather moderate at the same time as the arterioles dilated CFC increased with 5 HT administration but the rise was moderate

Noradrenaline Infusion of noradrenaline was followed by a pronounced constriction of resistance and capacitance vessels a net transcapillary absorption of fluid and a marked rise of CFC When the infusion was stopped a marked post infusion hyperemia appeared lasting for several minutes and yielding blood flow values comparable to those produced by *e g* PGE_1 This pattern of response including the marked elevation of CFC is identical to that ensuing from stimulation of the regional vasoconstrictor fibres as earlier shown (Öberg and Rosell 1967) and confirmed repeatedly in the present study (Table I) It is interesting to note that isoprenaline did not produce such a marked CFC increase as did noradrenaline This effect is thus largely due to *α* receptor stimulation This is further supported by the finding that α receptor blocking agents reduces the CFC increases upon sympathetic nerve stimulation (Öberg and Rosell 1967)

Sympathetic stimulation during infusion of vasodilator drugs To analyze the possible mechanisms behind the marked increase in CFC during vasoconstrictor fibre stimulation a series of experiments were performed in which the regional vasomotor fibres were stimulated during periods of infusions of the vasodilator drugs The drugs were administered in an amount to produce maximal or close to maximal flow values in the preparation It was assumed that an almost maximal relaxation of the vascular smooth muscles and therefore also of the precapillary sphincters was then at hand The stimulation were applied when a stable vasodilator response was established In some experiments the blood flow through the preparation was maintained constant throughout the experimental period by adjustments of a screw clamp on the arterial inflow catheter In this way shifts in precapillary sphincter tone due to alterations of the transmural pressure during the subsequent stimulation could be avoided The results of these experiments are demonstrated in Fig 4 where per cent changes (from prestimulatory control of CFC and blood flow produced by vasoconstrictor fibre stimulation during drug infusion have been plotted against each other It can be seen that stimulation of the vasomotor fibres during *infusions of* PGE_1 acetyl

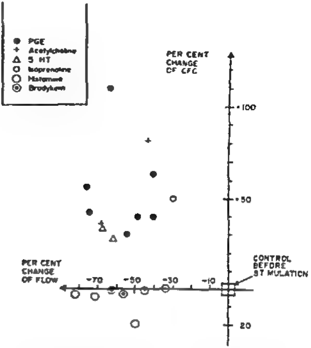

Fig 4 Effects of vasomotor fibre stimulation performed during periods of arterial infusions of various vasodilator drugs (different symbols) on CFC and blood flow in a subcutaneous adipose tissue preparation —The effects are expressed as per cent changes of prestimulatory control

choline 5 HT and isoprenaline can further increase CFC significantly despite the fact that the precapillary sphincters were supposedly already maximally dilated prior to the stimulation However when the vasoconstrictor fibres were stimulated during infusions of histamine or bradykinin, which *per se* produced very marked increases of CFC no further increases of CFC were obtained if anything CFC rather decreased

The finding that stimulation of the vasomotor fibres produced a marked increase of CFC even in situations when the precapillary sphincters were presumably maximally dilated seems to indicate that the increase in CFC during sympathetic stimulation is not only due to sphincter relaxation but also to some other factor possibly an increased permeability of the capillary membrane

Discussion

Of the vasodilator drugs used in the present study histamine and bradykinin produced the highest flow values *i e* 35—45 ml/100 g/min Bradykinin and histamine infusions were as it happened performed on tissue preparations with the lowest weights in the present series These preparations showed as earlier mentioned, the h[illegible] resting blood flow and CFC values when calculated on a weight basis, [illegible] therefore exhibit higher values for blood flow even in the maximally d[illegible]

Moreover, the per cent increase of flow, produced by bradykinin and histamine did not exceed those found with other drugs It is thus possible that the lower maximal blood flow values, of approximately 25 ml/100 g/min, obtained with PGE_1 isoprenaline etc (see Table I) also represent a maximal vasodilatation This latter flow value agrees with those obtained with vasomotor fibre stimulation after α receptor blockade and those found during the hyperemia following vasoconstrictor fibre stimulation (Ngai, Rosell and Wallenberg 1966, Öberg and Rosell 1967)

Administration of vasodilator drugs always produced an increase of CFC The maximal rise was moderate with PGE_1, acetylcholine, isoprenaline and 5 HT and amounted to a maximum of 40—45 per cent above resting control The CFC increased roughly in parallel with the blood flow, and was therefore probably due to a relaxation of the precapillary sphincters which would result in an increased number of patent capillaries available for exchange The relation between blood flow and CFC obtained with the mentioned drugs (Fig 2) compares well with that seen with vasomotor nerve stimulation after blockade of α adrenergic receptors and with that seen during poststimulatory hyperemia (Öberg and Rosell 1967) With histamine and bradykinin CFC increased considerably more, reaching maximal values of more than two times the control These two drugs are known to increase capillary permeability in other vascular beds (*e g* Spector 1958, Elliot, Horton and Lewis 1960)

The capacitance responses to the various drugs were usually small This is probably due to the fact that the veins show very little basal tone after denervation (Folkow and Öberg 1961) and were therefore initially almost maximally dilated The recorded *venous responses are therefore at least partly due to passive distension of the veins* when prevenous vessels dilate 5 HT showed a dissociation with regard to its effect on resistance and capacitance vessels Thus dilatation of the resistance vessels was accompanied by a constriction of the veins

From the available data some characteristics of adipose tissue circulation can be established and compared with other vascular circuits as *e g* skeletal muscle (see also Mellander and Johansson 1968) Maximal blood flow in adipose tissue (25—34 ml/100 g × min) is less than half of the corresponding value in skeletal muscle This seems to imply smaller dimensions of the arterial system in adipose tissue On the other hand maximal values for CFC produced by vasodilator drugs which *do not appear to effect membrane permeability* are if anything larger than in skeletal muscle The density of the capillary network in adipose tissue seems to be extensive as has also been shown in morphological studies (Gersh and Still 1945 This indicates that favourable conditions for capillary exchange are at hand in subcutaneous adipose tissue

Prostaglandins of the E series have been found to be potent inhibitors of lipolysis both *in vitro* and in intact animals and man (for review see Bergstrom Carlson and Weeks 1968) Dose-response characteristics for this action of PGE_1 in the present adipose tissue preparation have been reported by Fredholm and Rosell (1969) Shaw and Ramwell (1968) recently demonstrated the formation of prostaglandin like material in epididymal fat pads of the rat under conditions in which lipolysis was

stimulated A role has been proposed for the prostaglandins as physiological feedback regulators of lipolysis Therefore it appears possible, that the marked increase in CFC upon sympathetic nerve stimulation which is characteristic for adipose tissue might be the result of released prostaglandin exerting its vasodilator effect However the data do not seem to suport this hypothesis Thus the marked increase of CFC produced by nerve stimulation cannot be mimicked even by infusions of large doses of PGE_1

Histamine is known to be present in adipose tissue in significant amounts (Stock and Westermann 1963) and has also been shown to stimulate lipolysis in dog adipose tissue (Fredholm Meng and Rosell 1968 Ballard and Rosell 1969) Histamine might therefore be involved in the regulation of metabolic as well as circulatory events in adipose tissue Whether histamine does play such a physiological regulatory role in adipose tissue cannot yet be decided It seems unlikely however that histamine release is the cause of characteristic CFC increase following sympathetic nerve stimulation Thus this response remained when mepyramine maleate had been given in such amounts (1 mg) as to completely abolish the vascular effects of histamine and after prior treatment with compound 48/80 to deplete the tissue of available mast cell histamine (unpublished observations)

The effect of infusion of noradrenaline was directly comparable to that produced by nerve stimulation (Fig 2) Moreover the effects of isoprenaline were similar to those produced by sympathetic nerve stimulation after adrenergic α blockade (Öberg and Rosell 1967) Since the effects of nerve stimulation can be mimicked very well by adrenergic receptor agonists it is reasonable to assume that noradrenaline released from sympathetic nerve endings is the mediator of the vascular readjustments following sympathetic nerve stimulation

The high CFC obtained during sympathetic stimulation may be ascribed to a dilatation of precapillary sphincters and/or to an increased permeability of the capillary membrane In an earlier study (Öberg and Rosell 1967) the first mentioned explanation was considered to be the most likely one mainly because permeability changes with sympathetic nerve stimulation has not been described earlier in any other vascular bed The present experiments indicate that sphincter relaxation is a less likely cause of the increased CFC during sympathetic nerve stimulation Thus the infusion of vasodilator substances presumably causing maximal dilatation of precapillary sphincters never increased CFC to the values obtained during nerve stimulation Moreover nerve stimulation was capable of increasing CFC significantly even in situations when the precapillary sphincters were presumably already maximally dilated by vasodilator substances Recent studies by Linde and Rosell (1970) in which the clearance of Xe^{133} and NaI^{125} from a depot in the subcutaneous adipose tissue was followed during constant blood flow conditions show that sympathetic nerve stimulation produced a decrease in the isotope disappearance rate Apparently then the capillary surface area available for diffusion was if anything reduced during stimulation presumably due to constriction of precapillary sphincters This is in accordance with the suggestion by Rosell 1966 that vasomotor adjustments follow

ing sympathetic nerve stimulation, impairs the venous outflow of FFA from adipose tissue

Infusions of drugs, which presumably affects the porosity of the membrane, *e g* histamine and bradykinin, were the only means to produce CFC values of the same magnitude as obtained with sympathetic nerve stimulation Moreover, sympathetic stimulation during infusion of histamine or bradykinin did not increase CFC further, as was the case when stimulation was superimposed on the infusions of drugs like PGE_1, acetylcholine etc In fact, CFC rather tended to decrease It is therefore possible that in subcutaneous adipose tissue sympathetic stimulation induces an increased capillary permeability This possibility merits further study

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References

BALLARD K and S ROSELL The unresponsiveness of lipid metabolism in canine mesenteric adipose tissue to biogenic amines and to sympathetic nerve stimulation *Acta physiol scand* 1969 77 442—448

...ROSTROM S L A CARLSON and J R WEEKS The prostaglandins A family of biologically active lipids *Pharmacol Rev* 1968 20 1—48

...BOLD A B FOLKOW I KJELLMER and S MELLANDER Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient *Acta physiol scand* 1963 57 180—192

...LIOT D D E W HORTON and G P LEWIS Actions of pure bradykinin *J Physiol* (Lond) 1960 *153* 473—480

...LKOW B O LUNDGREN and I WALLENTIN, Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 57 270—283

...LKOW B and B ÖBERG Autoregulation and basal tone in consecutive vascular sections in the skeletal muscles in reserpine treated cats *Acta physiol scand* 1961 53 105—113

...EDHOLM B B H C MENG and S ROSELL Release of free fatty acids from canine subcutaneous adipose tissue by histamine and compound 48/80 *Life Sci* 1963 7 1209—1215

...EDHOLM B B and S ROSELL Metabolic effects of prostaglandin E_1 in canine adipose tissue in situ *Acta physiol scand* 1968 74 6A

...ERSH J and M A STILL Blood vessels in fat tissue Relation to problems of gas exchange *J exp Med* 1945 *81* 219 232

...NDE B and S ROSELL To be published

LINDGREN P An improved method for drop recording of arterial or venous blood flow *Acta physiol scand* 1958 42 5 11

MELLANDER S and B JOHANSSON Control of resistance exchange and capacitance functions in the peripheral circulation *Pharmacol Rev* 1968 29 117 196

NGAI S H S ROSELL and L R WALLENBERG Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs *Acta physiol scand* 1966 68 397—403

ÖBERG B and S ROSELL Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue *Acta physiol scand* 1967 71 47 56

ROSELL S, Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation *Acta physiol scand* 1966 67 343—351

SHAW, J E and P W RAMWELL Release of prostaglandin from rat epididymal fat pad on nervous and hormonal stimulation *J biol Chem* 1968 243 1498—1503

SPECTOR W G, Substances which affect capillary permeability *Pharmacol Rev* 1958 10 475—508

STOCK, K and E WESTERMANN Concentrations of norepinephrine serotonin and histamine and of amine metabolizing enzymes in mammalian adipose tissue *J Lipid Res* 1963 4 297—303

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Releasing Effect of Calcium and Phosphate on Catecholamines, ATP, and Protein from Chromaffin Cell Granules

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Abstract

LISHAJKO, F *Releasing effect of calcium and phosphate on catecholamines, ATP and protein from chromaffin cell granules* Acta physiol scand 1970 79 575—584

Addition of Ca^{2+} 3 mM to isolated bovine adrenal medullary granules incubated in 130 mM K phosphate buffer pH 6 8—7 2 causes a rapid initial release of noradrenaline (NA), adrenaline (A) ATP and soluble protein The effect is increased by addition of 1—6 mg/ml of RNA and in[illegible]
phosphate ions [illegible]
tion of fresh[illegible]
of $CaHPO_4$ or [illegible]
effective together with 1—6 mg/ml of RNA In the absence of phosphate ions Ca^{2+} and Mg^{2+} as well as the divalent ions Ba^{2+} and Sr^{2+} inhibited the release The catecholamine releasing effect of Ca^{2+} in the presence of phosphate is reduced by 2 mM ATP and almost completely prevented by ATP Mg^{2+} It is suggested that the increased outflow of granular contents is due to membrane alterations induced by Ca phosphate

The stimulating effect of acetylcholine (ACh) on the secretion of catecholamines (CA) from the isolated perfused adrenal gland requires calcium and is enhanced by Ba^{2+} and Sr^{2+} (Douglas and Rubin 1961 1963 Philippu and Schumann 1962 Douglas and Rubin 1964) Douglas and Poisner (1962) have also reported that ^{45}Ca is taken up into the chromaffin cells during stimulation and depolarization of the chromaffin cells by ACh

The ACh induced secretion also includes ATP (as metabolic AMP) (Douglas *et al* 1965 Stjarne 1964) and soluble protein (Banks and Helle 1965 Kirshner *et al* 1966 Blaschko *et al* 1967 and Schneider *et al* 1967

An approximately parallel release of CA and soluble protein has also been shown to occur in isolated adrenal medullary granules Lishajko 1969 b even after partial inhibition of release by ATP Mg

In the present communication the importance of phosphate ions for the release of CA ATP and soluble protein by Ca^{2+} is demonstrated and also that the effect is enhanced by RNA and inhibited by Mg^{2+} ATP and ATP Mg

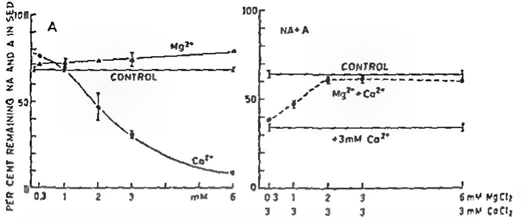


Fig 1 (Left) Isolated adrenal medullary granules incubated in 130 mM K phosphate pH 6 8—7 2 for 60 min at 37° C without and with addition of 0 3—6 mM $MgCl_2$ or $CaCl_2$ or in combination (right) Ordinate per cent remaining NA and A of original amount in sediment after incubation Abscissa concentration of added ions Each point is the mean of 2—7 expts

Methods

Bovine adrenal glands were obtained from the slaughter house and transported at ice tempera ture to the laboratory within 1 hr after the death of the animals All the subsequent prepara tions were made at 2—5 C The adrenal medulla was homogenized according to Potter Elve hjem in 270 mM sucrose or in 130 mM K phosphate buffer pH 6 8—7 5 (1 g/10—20 ml medium The suspension was centrifuged at 1000 × g for 8—10 min and the supernatant recentrifuged at 50 000 × g for 20—30 min The tube containing the high speed sediment was washed with ice-cold 270 mM sucrose or 130 mM K phosphate buffer and the sediment re suspended in fresh medium The final suspension of about 25 mg wet weight granules in an 1 ml incubation tube contained 178 ± 13 µg NA and 322 ± 19 µg A (M ± S E M, $n = 13$) Incubation was performed in a water bath without shaking at 37° C After centrifugation the walls of the tube were washed with cold incubation fluid the walls wiped dry, and the sedi ment extracted with 0 5 ml 0 4 N perchloric acid After addition of 5 ml water, the precip tate was spun down by centrifugation 5—10 min at 15,000 × g NA and A were estimated fluori metrically Euler and Lishajko 1961 ATP was estimated by the luciferase method of Strehler and Totter 1954 The protein was determined by Lowry *et al* (1951 Bovine albumine Sigma Chem Co and ATP were used as references RNA (from yeast qual 1) was obtained from Theodor Schuchardt or from Sigma Chemicals (type XI)

Results

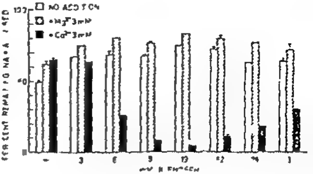
1 *Effect of Ca^{2+} and Mg^{2+} in phosphate media*

As shown in Fig 1 addition of Ca^{2+} (chloride or acetate) in concentrations of 2—6 mM to the granule suspension in phosphate medium strongly increases the release of A whereas 0 3—1 mM Ca^{2+} lacked effect The effect on NA does not differ from that on A The releasing effect of Ca^{2+} is completely blocked by 2 mM Mg^{2+} and markedly inhibited by 1 mM (Fig 1) Mg^{2+} alone has a slightly inhibitory effect

While 2—6 mM Ca^{2+} caused a precipitate in the phosphate medium this was not seen with 0 3—1 mM Ca^{2+} or with Mg^{2+} 0 3—6 mM

Addition of 3 µmoles per ml of calcium hydrogen phosphate ($CaHPO_4$) or pyrophosphate $Ca_2P_2O_7$ had no effect on the release of amines in K-phosphate medium

Fig 2 Isolated adrenal medullary granules incubated in 270 mM sucrose pH 6 7—6 8 in sucrose partially replaced with equiosmolar 130 mM K phosphate buffer pH 6 8, and in 130 mM K phosphate without and with addition of 3 mM $MgCl_2$ or $CaCl_2$ Ordinate per cent remaining NA+A of original amount in sediment after incubation Abscissa mM K phosphate buffer Each point is the mean of 2—7 expts



In the absence of phosphate both Ca^{++} or Mg^{++} retard the release of NA and A Thus no releasing effect of Ca^{2+} was observed in sucrose, NaCl or KCl media However, partial replacement of a sucrose medium by equiosmolar K phosphate gradually increases the release of NA and A with a maximum effect at about 20 mM (Fig 2) At higher K phosphate concentrations the effect was less pronounced The inhibitory Mg^{2+} effect was not changed by the replacement of sucrose by phosphate

2 *Effect of temperature on the effects of Ca^{2+} and Mg^{2+}*

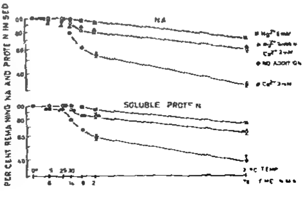
In order to study the influence on the Ca^{++} releasing effect of temperature this was gradually increased from 4° to 37° C in the incubation medium As seen in Fig 3 the effect of Ca^{++} is negligible up to 25° C and then rapidly increases both for NA and soluble protein until approximately the same release rate as in the control is reached The effect on A release is the same as for NA

The Ca^{++} effect is completely prevented by 2—6 mM Mg^{2+} 6 mM Mg^{2+} alone retards the release of the amines as well as soluble protein (*cf* Fig 1)

3 *Ca^{++} induced release of ATP and soluble protein*

The Ca^{2+} induced release of NA and A is accompanied by a release also of ATP and soluble protein As seen in Fig 4 the release is approximately parallel for these

Fig 3 Bovine adrenal medullary granules incubated in 130 mM K phosphate buffer pH 7 2 with additions as indicated. Temperature raised from +4° C to 37° C in 18 min Ordinate per cent remaining NA and soluble protein of original amount in sediment. Abscissa temperature and time interval Each point is the mean of 1—3 expts



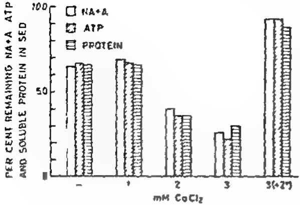


Fig 4 Adrenal medullary granules incubated in 130 mM K phosphate buffer pH 7 2, for 60 min at 37° C alone and with addition of 1—3 mM $CaCl_2$ Ordinate per cent remaining NA+A, ATP and soluble protein of original amount in sediment after incubation Abscissa mM $CaCl_2$ Columns in the right Incubation at 2° C

constituents of the granules on addition of 2 and 3 mM Ca^{2+} to a phosphate medium At +2° C the effect of Ca^{2+} is negligible

In unbuffered 270 mM sucrose Mg^{2+} or Ca^{2+} retard the release of soluble protein as well as of CA and ATP from the granules After partial replacement of the sucrose by 130 mM K-phosphate the releasing effect of Ca^{2+} appeared with a maximum at about 10—30 mM phosphate

Effect of Ca^{2+} at different amine amounts

While the release rate of CA in controls was not influenced by the amounts of granules used the releasing effect of 3 mM Ca^{2+} in the presence of phosphate was markedly reduced above 400 µg CA (Table I) suggesting a kind of stoichiometric relationship between the releasing factor and the granules

5 *Releasing effect of CA-phosphate precipitate during different conditions*

Since the Ca^{2+} concentrations which release NA and A also cause a precipitation in a K-phosphate medium the following experiments were made in order to study if newly formed Ca-phosphate in the incubation medium as such influences the release rate

TABLE I Bovine adrenal medullary granules incubated in 130 mM K-phosphate buffer pH 7 2 60 min at 37° C alone and with 1 and 3 mM $CaCl_2$ The table shows µg granule-bound amines in 8 ml tube before incubation, and per cent released NA and A of original amount in sediment

Added amines in one tube µg		Per cent NA and A released			
Amines in µg		No addition		+3 mM Ca^{2+}	
NA	A	NA	A	NA	A
35	[illegible]	34	33	97	93
77	130	[illegible]	29	96	[illegible]
155	262	32	28	70	71
240	406	33	28	70	71
321	527	32	28	67	66

TABLE II Isolated bovine adrenal medullary granules incubated in 130 mM K phosphate buffer pH 7.2, 60 min at 37° C, 0.3, 0.5, 0.75 and 1 mM $CaCl_2$ and RNA 1, 2, 4 and 6 mg/ml added as indicated. The table shows per cent remaining Na + A of original amount in sediment. Mean of 2 expts

Ca^{++} mM	0.3 NA + A	0.5 NA + A	0.75 NA + A	1 NA + A
No Addition	63	62	62	62
RNA 1 mg/ml	63	[illegible]	57 (−	53 (−
2	63	59 (−)	56 (−	42 −
4	58 (+)	52 (+)	37 −	25 − −
[illegible]	48 (+)	36 −	23 − −	15 − − −

(−) − − − + + + indicate degree of flocculation observed in model experiments without granules in 130 mM K phosphate buffer pH 7.2 after 60 min at 37° C

1 On addition of a medium containing freshly precipitated Ca-phosphate (24 μmoles $CaCl_2$ in 8 ml 130 mM K-phosphate buffer incubated for 15—30 min at 37° C) to a granule suspension an increased release rate of CA was observed amounting to about 60 per cent of that observed with $CaCl_2$ added direct to the granule suspension in phosphate

2 If the freshly precipitated Ca-phosphate was collected by centrifugation and added to the granule suspension, the release rate of CA was still increased but to a lesser extent about 45 per cent of that observed with direct addition of $CaCl_2$ suggesting that sedimentation and resuspension had altered the physico-chemical state and reactivity of the Ca-phosphate (*cf* Eanes and Posner 1968)

3 When the Ca-phosphate pellet after centrifugation had been stored for 24 hrs at 22° and allowed to dry, and subsequently added to the granule suspension it had no effect on the release of CA, indicating that it has been converted into a form lacking the binding affinity to the granules and hence the CA-releasing effect. Precipitation of Ca^{++} in K-phosphate medium is more efficient at 37° C than at 2° C. In all experiments the released NA and A could be recovered in the supernatant

6 *Influence of RNA on the release rate of CA, ATP and soluble protein*

While 0.3—1 mM Ca^{++} has no effect on the release rate of CA and causes no precipitation in K phosphate these concentrations became effective in the presence of RNA from 1—6 mg/ml (Table II). The table shows that the releasing effect of small concentrations of Ca^{++} depends on the amount of RNA in the incubation medium. Even under these conditions NA and A are accompanied by ATP and soluble protein. Table II shows good agreement with model and authentic experiments as regards flocculation and release of CA. No effect on the release rate of CA occurs when RNA 1—6 mg/ml alone or together with 1—3 mM Ca^{2+} was incubated in 270 mM sucrose + 30 mM TRIS maleic acid buffer pH 7.2 again demonstrating that phosphate ions are required. Granules incubated for 60 min at +2° C together with RNA 6 ml/ml + 1 mM Ca^{2+} lost only about 5 per cent of the CA

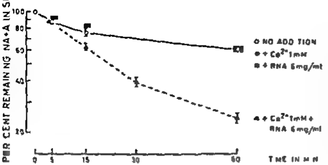


Fig 5 Bovine adrenal medullary granules incubated in 130 mM K phosphate buffer pH 7 2 at 37° C alone and with addition of Ca^{2+} 1 mM RNA 6 mg/ml and (Ca+RNA) combined Ordinate per cent remaining NA+A of original amount in sediment Abscissa incubation time in minutes

The effect of RNA and Ca^{2+} on the release rate of CA in 130 mM K-phosphate pH 7 2 is shown in Fig 5 From the figure it can be seen that RNA 6 mg/ml or 1 mM Ca^{2+} alone has no effect, while in combination they strongly increase the release of CA

Higher concentration of Ca^{2+}, 3 mM, together with RNA up to 6 mg/ml further increases the release of CA while 3 mM Mg^{2+} lacks enhancing effect even with RNA mg/ml (Fig 6)

Effect of ATP and ATP-Mg^{2+} on the action of Ca^{2+}

Previously it has been shown that ATP retards the release of NA and A in K-phosphate buffer as well as in sodium acetate medium (Lishajko 1969 a b) Since Mg^{2} antagonizes the Ca^{2} effect on amine release it appeared of interest to study whether ATP alone or together with Mg^{2+} acts in the same way

This was found to be the case Thus the Ca-induced amine release is partly blocked by ATP 2 mM and completely prevented by ATP-Mg^{2+} (Fig 7) The blocking effect of 2 mM ATP-Mg on the Ca-induced release is not overcome by RNA 6 mg/ml

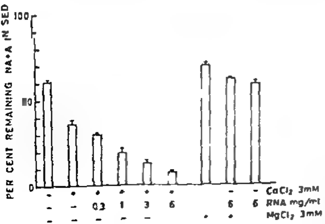


Fig 6 Bovine adrenal medullary granules incubated in 130 mM K phosphate buffer pH 7 2 for 60 min at 37° C Ordinate per cent remaining NA + A of original amount in sediment Abscissa additions as indicated

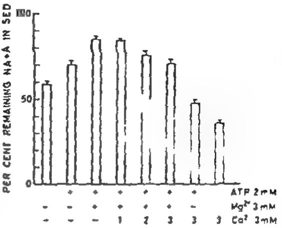


Fig 7 Isolated bovine adrenal medullary granules incubated for 60 min at 37° C in 130 mM K phosphate buffer pH 7 5 Ordinate per cent remaining NA+A of original amount in sediment Abscissa additions as indicated

8 *Effect of Ca^2, Mg^2, Ba^2 and Sr^2 ions*

On addition of the divalent cations Ca^{++}, Mg^{2}, Ba^{+}, Sr^{2} to a sucrose medium giving a concentration of 3 mM the amine release was retarded in all instances In the presence of phosphate only Ca^{++} had a strong releasing effect while Sr^{2} had a slight action (Table III) Addition of RNA 6 mg/ml did not increase the effect of 3 mM Sr^{2} or Ba^{+} but slightly reduced the retarding effect of 3 mM Mg^{+}

Addition of 3 mM Mn^{+} and Co^{2} caused a releasing effect in phosphate medium but this was not blocked by Mg^{2} and [illegible] therefore considered to be different in nature from that of Ca^{+} It was also observed that Mn^{+} caused a much stronger release effect than Ca^{+}

The specificity of the Ca^{+} effect is further brought about by the fact that Ba^{+} and Sr^{2}, which hardly influence the CA release in the presence of phosphate also cause a precipitation although this appears to differ in some respects from that of Ca^{+} (*cf* Eanes and Posner 1968)

TABLE III Bovine adrenal medullary granules [illegible] pH 6 7 [illegible] in 130 m [illegible] Sr^{2} Ba^{2} and A of [illegible]

Ions added 3 mM	in sucrose alone		sucrose +9 mM K phosphate		130 mM K phosphate		130 mM K phosphate +RNA [illegible] mg ml	
	NA	A	NA	A	NA	A	NA	A
addition	40	63	60	75	60	65	[illegible]	61
[illegible]	50	74	[illegible]	78	72	0	63	63
[illegible]	56	73	[illegible]	6	37	36	7	8
[illegible]	53	73	72	73	55	59	54	60
[illegible]	57	76	74	77	56	63	59	61

Discussion

The present results show that while Ca^{++} like several other divalent cations retard amine release in unbuffered sucrose medium its action is reversed in the presence of phosphate manifesting itself as a strong releasing action on CA ATP and soluble protein The somewhat smaller releasing effect in high phosphate concentrations suggests that the monovalent cations present interact with the Ca^{2+} in the system (*cf* Fig 2)

The effect of Ca^{++} is apparently in some way associated with a precipitation of Ca phosphate It is noteworthy, however, that Mg^{++} or ATP alone or in combination inhibit or prevent the Ca^{++} effect without affecting to any larger extent the precipitation Furthermore Sr^{2+} and Ba^{++} also cause precipitation in the phosphate medium but have only a small effect on the release not potentiated by RNA It is known however that Sr^{++} and Ba^{++} rapidly form a stable precipitate with phosphate as reported by Eanes and Posner (1968) The slight antagonistic effect of RNA upon the retarding effect of Mg^{++} may be due to a binding of these ions as shown by Wacker and Vallee (1959) (Table III)

In view of the inhibitory action of Mg^{++} on the CA release induced by Ca^{++} and phosphate in our experiments it is of interest to note that Douglas and Rubin (1963) have observed an antagonistic effect on the ACh induced CA release from the perfused adrenal gland by Mg^{2+}

The rapid initial fall of CA ATP and protein induced by Ca^{++} in K phosphate medium might be explained as an action of a calcium phosphate complex on the granule membrane altering its physico chemical state The nature of this complex is so far unknown but our experiments have shown that while the freshly produced precipitate still has a releasing action this is lost on ageing of the precipitate Interaction of Ca^{++} and other divalent ions with phosphoric groups has been reported for phospholipids by Shah and Schulman (1967) mononucleotides (Walaas 1958 Cohn and Hughes 1962 and nucleic acid (Wacker and Vallee 1959) In systems of this kind ATP Mg might compete with Ca^{++} for binding sites thereby stabilizing the membrane or preventing the postulated complex formation an effect which is not overcome by RNA

Since 0 3—1 mM Ca^{++} only in combination with RNA is able to increase the release rate RNA appears to interact with the release inducing Ca-complex On the other hand RNA is ineffective without Ca^{++} and phosphate ions It is unlikely that the Ca^{++} effect is unspecific in the K phosphate medium since it is small or absent below 25—30 The rapid fall of CA ATP and soluble protein caused by Ca^{++} can hardly be an osmotic effect since some effect was obtained in hypertonic K phosphate or after previous equilibration with Ca^{++} before granules were added to the media for incubation The relatively smaller release observed with Ca^{++} when large amounts of granules were incubated also suggests that it is not an osmotic effect but rather saturation of some binding site on the granule membrane

Hillarp (1960) and Philippu and Schumann (1962) suggested that in vivo

granules might secrete the content direct to the ce ap fo wed by diffus on into the extracellular space This mechanism of release v uld r ive apport if t Ca effect described in this paper also operates *in vivo*

On the other hand, if release occurs at the pla na membrane ot he l a proposed by De Robertis and Vaz Ferreira (1957), Banks and Helle 19 and Poisner (1966), Kirshner *et al* (1966) and Schneider *et al* (1967 it might be considered that Ca facilitates a fusion between granules and membrane through the mediation of intracellular phosphate i ns and RNA natively Ca^{2+} might reduce ionic repulsion between gran es d pla m m brane reducing negative charges only on one of the membranes wid the res granules fuse with the plasma membrane (*cf* Katz 1969 The rate of Ca tration through the cell membrane might then determine the release rate thr interaction of Ca^{2+} with intracellular phosphate and granules on the one hand ar plasma membrane on the other

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References

Banks P and K Helle The release of protein from the stimulated adrenal medulla *Biochem J* 1965 *97* 40C—41C.

Blaschko H R S Comline F H Schneider M Silver and A D Smith Secretion of a chromaffin granule protein chromogranin from the adrenal gland after splanchnic st mu lation *Nature* (Lond) 1967 *215* 58—59

Cohn M and Th R Hughes Jr Nuclear magnetic resonance spectra of adenosine di and triphosphate *J biol Chem* 1962 *237* 176—181

Douglas W W and R P Rubin The role of calcium in the secretory response of the adrenal medulla to acetylcholine *J Physiol* (Lond 1961 *159* 40—57

Douglas W W and A M Poisner On the mode of action of acetylcholine in evoking adrenal medullary secretion increased uptake of calcium during the secretory response *J Physiol* (Lond) 1962 *162* 385—392

Douglas W W and R P Rubin The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus secretion coupling *J Physiol* (Lond) 1963 *167* 288—310

Douglas W W and R P Rubin The effects of alkaline earths and other divalent cations on adrenal medullary secretion *J Physiol* (Lond 1964 *175* 231—241

Douglas W W A M Poisner and R P Rubin Efflux of adenine nucleotides from per fused adrenal glands exposed to nicotine and other chromaffin cell stimulants *J Physiol* (Lond) 1965 *179* 130—137

Douglas W W and A M Poisner Evidence that the secreting adrenal chromaffin cell releases catecholamine directly from ATP rich granules *J Physiol* Lond) 1966 *183* 236—248

Eanes E D and A S Posner Intermediate phases in the basic solution preparation of alkal ne earth phosphates *Calc Tiss Res* 1968 *2* 38—48

Euler U S v and F Lishajko Improved technique for the fluorimetric estimation of cate cholamines *Acta physiol scand* 1961 *51* 348—355

Hillarp, N A Different pools of catecholamines stored in the adrenal medulla. *Acta physiol scand* 1960 *50* 8—22

Katz B *The release of neural transmitter substances* In "The Sherrington Lectures X Liverpool University Press Printed in England by Hazell Watson & Viney Ltd. 1969

Kirshner N H J Sage W J Smith and A G Kirshner Release of catecholamines and specific protein from adrenal glands *Science* 1966 *154* 529—531

LISHAJKO F Influence of chloride ions and ATP Mg on the release of catech
isolated adrenal medullary granules *Acta physiol scand* 1969a 75 255—256

LISHAJKO F Release reuptake and net uptake of dopamine noradrenaline and a
isolated sheep adrenal medullary granules *Acta physiol scand* 1969b 76 15

LOWRY O H N J ROSENBROUGH A L FARR and R J RANDALL Protein measur
the Folin phenol reagent *J biol Chem* 1951 193 265—275

PHILIPPU A and H J SCHÜMANN Der Einfluss von Calcium auf die Brenzcatech
setzung *Experientia (Basel* 1962 *18* 138—140

ROBERTIS E DE and A VAZ FERREIRA Electron microscope study of the exc
chol-containing droplets in the adrenal medulla *Exp Cell Res* 1957 *12* 568

SCHNEIDER F H A D SMITH and H WINKLER Secretion from the adrenal
chemical evidence for exocytosis *Brit J Pharmacol* 1967 *31* 94—101

SHAH D O and J H SCHULMAN The ionic structure of lecithin monolayers *J*
1967 *8* 227—233

STJÄRNE L Studies of catecholamine uptake storage and release mechanisms *A*
scand 1964 *62* Suppl 228

STREHLER B L and J R TOTTER *Determination of ATP and related compo*
luminescence and other methods In "Methods of Biochemical Analysis Ed D
I 341—356

WACKER W E C and B L VALLEE Nucleic acids and metals I Chromium
nickel iron and other metals in ribonucleic acid from diverse biological sourc
Chem 1959 *234* 3257 3262

WALAAS E Stability constants of metal complexes with mononucleotides. *Act*
1958 *12* 528—536